# Studies on *in vivo* Behaviour and Perfused Liver Uptake of Cationic Liposomes in Rat

1998

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# Contents

# Preface

Liposomes have been studied over the past three decades as both biomembrane models and drug carriers. Liposomes have various advantages as drug carriers, being biodegradable, having low toxicity and being able to encapsulate hydrophilic, lipophilic and amphiphilic drugs. The encapsulation of biologically active substances into liposomes is useful in that biologically active substances can be protected from inactivation *in vivo* and the toxicity of anticancer drugs such as cytosine arabinoside and adriamycin can be decreased [1-3]. However, there are inevitable drawbacks in the use of liposomes *in vivo*. Liposomes are recognized as foreign substances and phagocytosed by cells of the reticuloendothelial system (RES). Their rapid removal from the circulation following intravenous administration is primarily due to phagocytosis by the Kupffer cells and macrophages of the spleen [4, 5]. Native liposomes are especially suitable for targeting diseases of the RES such as leishmaniasis and fungal infections [6, 7], but are undesirable for delivering drugs to other organs.

There are many factors affecting the fate of liposomes *in vivo* such as lipid composition, liposome size, surface charge, and lipid fluidity. For example, small unilamellar vesicles (SUVs) are taken up less rapidly than large ones [8]. Incorporation of cholesterol (Chol) into liposomes increases the stability of liposomes in the serum and decreases the clearance rate from the blood circulation [9]. Coating liposomes with polysaccharides, particularly ganglioside $G_{M1}$, can lead to a prolonged lifetime in the circulation due to low uptake by RES. Sialic acid plays an important role in the process of uptake of $G_{M1}$-liposomes by RES. However, $G_{M2}$ and $G_{M3}$, which have also a sialic group, have no such RES avoiding nature [10]. In another approach to avoiding phagocytosis by macrophages, the coating with polyethylene glycol (PEG) can prolong the circulation time of liposomes due to steric hindrance or increase liposomal surface hydrophilicity [11-14]. Cationic stearylamine-liposomes also remain in the blood longer than neutral or anionic liposomes (containing phosphatidylserine or phosphatidic acid) [15] and cationic liposomes containing a basic lipid, DOTMA (*N*[1-

(2,3-dioleyloxy)propyl]-*N,N,N*-triethylammonium chloride) and DOPE (dioleolylphsophatidylethanolamine) have been studied during the last 10 years as tools for the delivery of plasmid DNA and RNA into cells [16-18]. The administration of liposomes containing stearylamine, however, gives rise to convulsions [19-21], so that the stearylamine-containing liposomes are unsuitable for clinical use. Liposomal vectors formulated with various basic lipids and DOPE have been also reported to be toxic [22-24]

There is another problem in using liposomes as drug carriers. For the therapeutic use of liposomes, efficient encapsulation and retention of drugs is required before successful delivery can be achieved. However, little can be encapsulated into liposomes, since liposomes, especially multilamellar vesicles (MLVs) and small unilamellar vesicles (SUVs), have a low volume of entrapped aqueous space per mole of lipid. Reverse-phase evaporation[25] and pH gradient (or membrane potential) loading methods[26, 27] have been developed to entrap large amounts of substances into liposomes. However, these methods cannot be applied to entrap enzymes into liposomes, since the sonication in the reverse-phase evaporation procedure often inactivates the enzymes. Also, pH gradient loading can be applied to only small lipophilic molecules that can pass through the lipid membranes.

In this study, the author synthesized biodegradable monoesters of fatty acid with amino sugars to develop low toxic cationic liposomes that avoid uptake by RES. The author also tried to entrap superoxide dismutases (SODs) into cationic liposomes using an electrostatic attraction. Futhermore, the author investigated the *in vivo* behavior of cationic liposomes in rat and elucidated the mechanism of their uptake by the liver using a liver perfusion technique.

The results obtained in this work should provide valuable information for the design of improved formulations of liposome.



## Abbreviations

| | |
|---|---|
| $^3$H-CHE | [1,2(n)-$^3$H]-cholesteryl hexadecyl ether |
| Chol | cholesterol |
| DiI | 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate |
| DOPE | dioleolylphsophatidylethanolamine |
| DOTMA | (*N*[1-(2,3-dioleyloxy)propyl]-*N,N,N*-triethylammonium chloride |
| DPTMP | 1,2-dipalmitoyl-3-trimethylammonium propane |
| EPC | egg yolk L-α-phosphatidylcholine |
| HEPES | 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid |
| LacCer | lactosylceramide |
| MLVs | multilamellar vesicles |
| PBS | phosphate buffered saline |
| PEG | polyethylene glycol |
| PGal | methyl-6-*O*-palmitoyl-D-galactopyranoside |
| PGalN | methyl-2-amino-2-deoxy-6-*O*-palmitoyl-D-galactoside |
| PGlc | methyl-6-*O*-palmitoyl-D-glucopyranoside |
| PGlcN | methyl-2-amino-2-deoxy-6-*O*-palmitoyl-D-glucoside |
| PMan | methyl-6-*O*-palmitoyl-D-mannopyranoside |
| PManN | methyl-2-amino-2-deoxy-6-*O*-palmitoyl-D-mannoside |
| RES | reticuloendothelial system |
| REVs | reverse-phase evaporation vesicles |
| SA | stearylamine |
| SDS-PAGE | SDS-polyacrylamide gel electrophoresis |
| SOD | superoxide dismutase |
| SUVs | small unilamellar vesicles |
| TMS | tetramethylsilane |
| TNS | 2-*p*-toluidinylnaphthalene-6-sulfonate |
| Tris | tris(hydroxymethyl)aminomethane |
| $VET_{200}$ | vesicles with a diameter of 200 nm prepared by extrusion technique |
| WSC | 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, hydrochloride |
| XOD | xanthine oxidase |

| | |
|---|---|
| Control-L | $VET_{200}$ composed of EPC:Chol (60:20 in molar ratio) |
| PGlcN-L | $VET_{200}$ composed of EPC:Chol:PGlcN (60:20:20) |
| PGalN-L | $VET_{200}$ composed of EPC:Chol:PGalN (60:20:20) |
| PManN-L | $VET_{200}$ composed of EPC:Chol:PManN (60:20:20) |
| PGlc-L | $VET_{200}$ composed of EPC:Chol:PGlc (60:20:20) |
| PGal-L | $VET_{200}$ composed of EPC:Chol:PGal (60:20:20) |
| PMan-L | $VET_{200}$ composed of EPC:Chol:PMan (60:20:20) |
| PGlcN10-L | $VET_{200}$ composed of EPC:Chol:PGlcN (70:20:10) |
| PGlcN20-L | $VET_{200}$ composed of EPC:Chol:PGlcN (60:20:20) |
| PGlcN30-L | $VET_{200}$ composed of EPC:Chol:PGlcN (50:20:30) |
| PGlcN40-L | $VET_{200}$ composed of EPC:Chol:PGlcN (40:20:40) |
| SA5-L | $VET_{200}$ composed of EPC:Chol:SA (75:20:5) |
| SA10-L | $VET_{200}$ composed of EPC:Chol:SA (70:20:10) |
| SA15-L | $VET_{200}$ composed of EPC:Chol:SA (65:20:15) |
| SA20-L | $VET_{200}$ composed of EPC:Chol:SA (60:20:20) |



# Chapter 1

## Physicochemical properties of cationic liposomes modified with novel synthetic aminoglycolipid and their application to entrapment of SOD

Superoxide dismutases (SODs) are metalloenzymes that defend against oxidative stress through the decomposition of superoxide radicals, especially superoxide anion ($O_2^-$). SODs should be useful as drugs that suppress inflammatory disease and ischemic myocardial injury. However, their rapid renal filtration and disappearance from the circulation (their half-lives in the circulation are <6 minutes) limit therapeutic use, since molecular mass of SODs range from 32000 to 80000 [28]. Therefore, the circulation lifetime can be greatly increased by coupling SODs to various polymers such as dextran, poly(ethylene glycol) (PEG), etc. with a view to increasing molecular weight [29]. However, this chemical modification causes loss of protein activity.

The encapsulation of biologically active substances into liposomes is useful in that these substances can subsequently be protected from inactivation *in vivo* and also the toxicity of anticancer drugs such as cytosine arabinoside and adriamycin can be decreased [1-3, 30]. Turrens *et al.* demonstrated that, when SODs were entrapped in liposomes, their half-lives in circulating blood increased from 6 minutes to 4.2 hours [28, 31]. For the therapeutic use of liposomes, efficient encapsulation and retention of drugs is required before successful delivery can be achieved. Liposomes, especially multilamellar vesicles (MLVs) and small unilamellar vesicles (SUVs), have a low volume of entrapped aqueous space per mole of lipid. Therefore reverse-phase evaporation [25] and pH gradient (or membrane potential) loading methods [26, 27] have been developed to entrap large amounts of substances into liposomes. However, these methods cannot be applied to entrap enzyme into liposomes, since contact with organic solvent and/or the sonication in the reverse-phase evaporation procedure inactivate the enzyme. Also, pH gradient loading can be applied only to small lipophilic mol-

ecules that can pass through the lipid membranes.

Cu, Zn-SOD from bovine erythrocytes has an isoelectric point at 4.95 and thus bears a negative charge at physiological pH. The author tried to entrap SODs into cationic liposomes using an electrostatic attraction. Stearylamine (SA) is the most popular basic lipid used to give positive charge to liposomes. However, SA is difficult to apply therapeutically because of its toxicity [19-21]. Therefore, the author synthesized a biodegradable monoester of fatty acid with glucosamine, and tried to entrap SODs. This chapter deals with the physico-chemical properties of newly synthesized aminoglycolipid-modified liposomes and the entrapment efficiency of SODs into liposomes at a constant lipid concentration by various liposome preparation methods. The goal is to prepare liposomes containing a large amount of biologically active substances which can survive long-term without loss of activity.

## 1-1 Materials and Methods

*Materials*

Methyl-2-amino-6-palmitoyl-D-glucoside (PGlcN, Chart 1-5) was synthesized as described below according to the reaction scheme shown in Chart 1. Egg yolk L-α-phosphatidylcholine (EPC) was obtained from Asahi Kasei Co. Ltd. (iodine value 65, Tokyo, Japan). Superoxide dismutase (Cu, Zn-SOD, 3000 units/mg) from bovine erythrocytes, xanthine oxidase (XOD) from buttermilk, stearylamine (SA), and 2-*p*-toluidinylnaphthalene-6-sulfonate (TNS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Calcein (3,3'-bis[N,N-bis(carboxymethyl)aminomethyl]-fluorescein) was supplied by Dojin (Kumamoto, Japan). All other chemicals from Wako Pure Chemical Ind. Ltd. (Osaka, Japan) were of special grade, and cholesterol (Chol) was used after recrystallization from ethanol. Water was glass distilled twice.



*Synthesis of 2-amino 6-palmitoyl glucoside*

*Synthesis. 2-(N-Benzyloxycarbonyl)amino-2-deoxy-D-glucose* **(2)**: Benzyl chloroformate (7 g) was added dropwise at 5 °C to a mixed solution of water (90 ml) and methanol (50 ml), containing glucosamine **1** (8.3 g) and sodium hydrogencarbonate (6 g). The mixture was stirred well for 6 hours at room temperature, and then washed with water, to give compound **2** (52 %) (step a in Chart 1).

*Methyl-2-(N-Benzyloxycarbonyl)amino-2-deoxy-D-glucoside* **(3)**: A mixture of compound **2** (3.5 g) and p-toluenesulfonic acid (0.3 g) in anhydrous methanol (200 ml) was refluxed for 60 hours. After neutralization with sodium hydrogencarbonate, the mixture was filtered. The filtrate was evaporated and crystallized from ethyl acetate to give compound **3** (3.2g, 87 %) (step b in Chart 1)

*Methyl-2-(N-Benzyloxycarbonyl)amino-2-deoxy-6-O-palmitoyl-D-glucoside* **(4)**: A mixture of compound 3 (10 g) and palmitoyl chloride (7.7 g) in pyridine (60 ml) was stirred overnight. The reaction mixture was poured into 10% hydrochloric acid and extracted with ethyl acetate. The organic layer was washed with saturated sodium chloride solution, dried over sodium sulfate, and evaporated to afford a residue, which was chromatographed over silica gel. Elution with chloroform-ethyl acetate (95:5) gave compound 4c at a 24% yield (step c in Chart 1)

*Methyl-2-amino-2-deoxy-6-O-palmitoyl-D-glucoside* **(5)**: Compound 4 (1.2 g) in 30 ml of methonal-ethyl acetate (1:1) was stirred with 5 % palladium carbon in an atmosphere of hydrogen overnight. After removal of the catalyst by filtration, the solvent was evaporated to leave an oil which was purified by column chromatography over silica gel with chloroform-methonal (99:1) as an elute to give compound **5 (PGlcN)** (step d in Chart 1)

The synthesized compound was characterized by measurement of its $^1$H-NMR spectrum in DMSO-$d_6$ using TMS as a reference on a spectrometer (JMN-GX400, JEOL), by elemental analysis, infrared and mass spectra. The positions of the esters were determined by 2D H-H COSY. $^1$H NMR 0.86 (t, J = 6.0 Hz, 3H), 1.24 (b, 24H), 1.51 (m, 2H), 2.29 (t, J = 7.2 Hz, 2H), 2.40 (m, 1H), 3.10 (m, 2H), 3.26 (s, 3H), 3.53 (m,

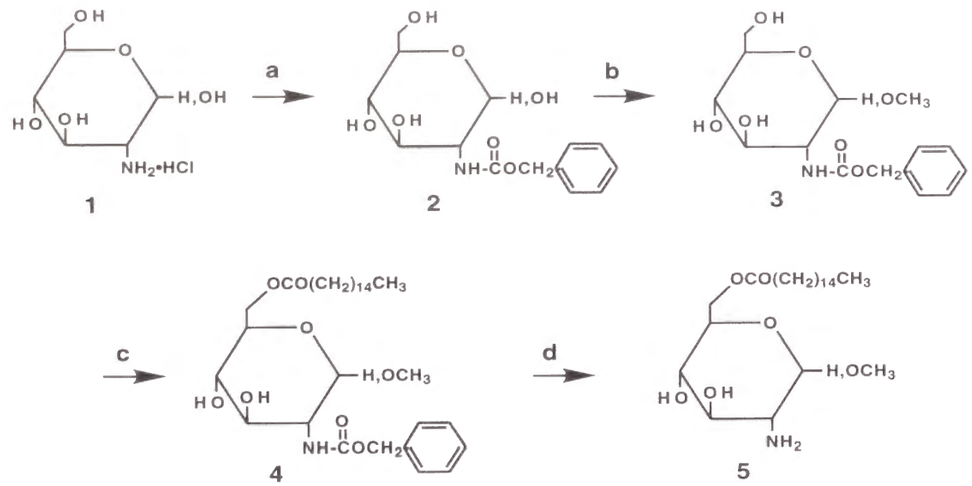


**Chart 1**

1H), 4.04 (dd, J = 6.6 Hz, J = 6.8 Hz, 1H), 4.30 (d, J = 10.6 Hz, 1H), 4.51 (d, J = 3.4 Hz, 1H), 5.00 (m, 1H), 5.15 (m, 1H) The cmc value of PGlcN was 0.71 μM using a TNS fluorescence technique [32].

*Distribution of synthetic aminoglycolipid into phosphatidylcholine/cholesterol membrane*

Multilamellar vesicles (MLVs) containing PGlcN were prepared in 10 mM HEPES/150 mM NaCl buffer (pH 7.4). The suspensions were then frozen with liquid $N_2$ and dissolved in a water bath at 25 °C [33]. These processes were repeated 5 times. The liposomal suspension was then ultracentrifuged (100,000 g x 1 hour), and the amount of hexosamine and phosphorous in the supernatant was determined by the Dische-Borenfreund method [34] and Bartlett method [35], respectively. The pellets were then resuspended with HEPES/150 mM NaCl buffer (pH 7.4). The suspensions were successively extruded 5 times each through polycarbonate filters with pore sizes of either 0.6 or 0.2 μm ($VET_{200}$: Vesicles with a diameter of 200 nm prepared by extrusion technique). The amounts of hexosamine, phosphorous and cholesterol in the liposomal suspension were determined by the Dische-Borenfreund method, Bartlett method and Cholesterol Test Wako, respectively.



*Hemolytic activities of positively charged liposomes*

Stearylamine enhances the permeability of biomembrane and damages the cells [36, 37]. Therefore, the hemolytic activity of positively charged liposomes (incorporation of 20 mol % PGlcN or SA) was investigated to compare SA and PGlcN. Liposomes were prepared by extrusion in 10 mM HEPES/150 mM NaCl (pH 7.4) buffer ($VET_{200}$). Rat erythrocytes were dispersed in the same buffer, mixed with a liposomal suspension, then incubated at 37 °C. After 1 hour, the suspension was centrifuged (2,000 g x 1 minute), and the absorbance of supernatants was measured at 540 nm on a spectrophotometer (UV-265FW, Shimadzu Co., Japan). The absorbance corresponding to 100 % hemolysis was determined by adding 5 ml of water to 1 ml of the erythrocyte suspension. The lipid concentration was determined by Bartlett's method [35].

*Toxicity of liposomes*

Since SA-liposomes show toxicity [21], the lethal dose of positively charged liposomes (incorporation of 20 mol % PGlcN or SA) was investigated. Liposomes ($VET_{200}$) were injected into the tail vein of ten male ddy mice weighing 25 g and the survival was observed immediately after the injection.

*SOD activity assay*

The SOD activity was determined by the nitrite method with a small modification [38]. The sample (0.1 ml), reagent A (0.2 ml, 65 mM $KH_2PO_4$, 35 mM $Na_2B_4O_7$ and 0.5 mM DTPA), and reagent B (0.2 ml, 0.5 mM hypoxanthine and hydroxylamine-*O*-sulfonic acid) were mixed and incubated for 15 minutes at 37 °C. Reagent D (0.2 ml, 0.025 unit/ml XOD) was added to this mixture and incubated for 30 minutes at 37 °C. Thereafter the mixture was added to 2.0 ml of coloring reagent E (30 mM *N*-1-naphthylethylenediamine, 3 mM sulfanilic acid and 25 % acetic acid). The final solution was allowed to stand for 1 hour at room temperature and the optical absorption was measured at 550 nm. The 50 % inhibitory dilutions ($ID_{50}$) were obtained for different dilutions of samples; the amount of SOD in the samples was then calculated

from the $ID_{50}$ compared with the 50 % inhibitory concentration ($IC_{50}$) of the standard SOD solution, assuming no loss of the activity during liposomal preparation.

*Protein assay*

The SOD content was also measured by the Lowry method with a small modification [39].

*Preparation of SOD entrapped liposomes*

1) Hydration method

Multilamellar vesicles (MLVs) were prepared by extrusion [40]. The lipid mixture in chloroform (EPC:Chol:PGlcN or SA in the desired molar ratios) was dried in a rotary evaporator to form a lipid film on the wall of a round-bottomed flask. The thin lipid film was left under reduced pressure for at least 12 hours to completely remove the solvent. The dried lipid was then dispersed with a 100 μg/ml SOD solution, which contained 10 mM Tris-HCl/150 mM NaCl (pH 7.4) as a buffer of high ionic strength or 10 mM Tris-HCl/300 mM sorbitol (pH 7.4) as a buffer of low ionic strength. The suspensions were successively extruded 5 times each through polycarbonate filters with pore sizes of either 0.6 or 0.2 μm The total lipid concentration of liposomal solutions was kept constant at 10 mM.

Untrapped SOD was removed by gel filtration (Bio-Gel 1.5m, 2 cm x 35 cm, eluted with 10 mM Tris-HCl/150 mM NaCl (pH 8.4)). The liposomes containing SOD were assayed for SOD activity and the amount of phospholipid after the addition of Triton X-100. The addition of Triton X-100 hardly inhibited the SOD activity assay. The concentration of phosphatidylcholine was determined by Bartlett's method [35]. The trapping efficiency was expressed as a SOD g/lipid mol. Ten grams of SOD/mol lipid corresponded to 100% trapping efficiency.

2) Reverse-phase evaporation method

Reverse-phase evaporation vesicles (REVs) were prepared according to Szoka



and Papahadjopoulos [25]. The thin lipid films (20 μmol lipids) were dissolved in 6 ml of diethyl ether. Two milliliters of 100 μg/ml SOD solution was added to the lipid solutions and sonicated under nitrogen in a bath-type sonicator (BRANSON 2200) at 25 °C for 5 min. After evaporation at 350-400 mmHg to remove ether, the suspensions were vortexed, then again evaporated at 680-700 mmHg. The suspensions were then extruded 5 times through polycarbonate filters of a 0.2 μm pore size.

3) Freeze-thawing method

The " empty " liposomes ($VET_{200}$) were prepared in the buffer described above. The liposomal suspensions were added to SOD solutions (final SOD concentration 100 μg/ml, lipid concentration 10 mM). The suspensions were then frozen with liquid $N_2$ and dissolved in a water bath at 25 °C. These processes were repeated 5 times [33]. The suspensions were also extruded 5 times through polycarbonate filters of a 0.2 μm pore size.

4) Dehydration-rehydration method

The " empty " liposomes ($VET_{200}$) prepared in the buffer described above were frozen in liquid $N_2$ and lyophilized on a KYOWA vacuum engineering model RL-10NB overnight. The dry samples were rehydrated to their original volumes with 100 μg/ml SOD solutions (lipid concentration 10 mM) [41]. The suspensions were then extruded 5 times through polycarbonate filters of 0.2 μm pore size.

*Liposome size and zeta potential measurement*

The size of the liposomes was measured by dynamic light scattering on a Photal laser particle analyzer LPA-3100 connected to a photon correlater LPA-3000 (Otsuka Electronics Co., Osaka, Japan)

The zeta potentials of liposomes with a diameter of about 200 nm were calculated from their electrophoretic mobilities [42] in 10 mM Tris-HCl/ 0 mM NaCl (as a substitute for 300 mM sorbitol) or 10 mM Tris-HCl/150 mM NaCl buffer (pH 7.4) at 25

°C. The mobilities were measured by a Zetasizer 4 electrophoretic light scattering spectrophotometer using a ZET 5103 small capillary cell (Malvern Instruments, Worcs., UK).

*Stability of liposomes in serum*

The thin lipid films were hydrated with 1 mM calcein/10 mM Tris/150 mM NaCl buffer (pH 7.4). The suspensions were vortexed and extruded through polycarbonate filters as described above. Untrapped calcein was removed by gel-filtration (Bio-Gel A-1.5m, 10 mM Tris/150 mM NaCl buffer (pH 7.4) as an elute). One milliliter of the liposomal suspension was mixed with 4 ml of prewarmed rat serum and the mixture was incubated at 37 °C. The retention of calcein in liposomes was determined fluorometrically (excitation at 490 nm and emission at 520 nm) on a spectrofluorometer (RF-5000, Shimadzu Co., Japan). The percentage of retention was calculated from Eqn 1.

$$retention\,(\%) = \left(1 - \frac{F_1 - F_2 \times \frac{3.1}{3}}{F_3 \times \frac{3.1}{3}}\right) \times 100 \qquad (1)$$

where, $F_2$ and $F_1$ are the fluorescence intensity of calcein entrapped in liposomes with and without 1 mM cobalt chloride, respectively (Sawahara et al., 1991). $F_3$ is the fluorescence intensity of calcein after addition of 10 % v/v Triton X-100 corresponding to 100 % leakage. The factor, (3.1/3) is for volume correction.

## 1-2 Results and discussion

*Lipid analysis*

In the supernatant after ultracentrifugation of liposomal suspension (MLVs), hexosamine derived from free PGlcN was barely detectable (data not shown). This showed that the synthesized amino-glycolipid was stably incorporated into the EPC/cholesterol bilayer. Table 1-1 shows the analyzed lipid composition of the extruded



liposomes ($VET_{200}$). The lipid composition of the extruded liposomes was the same as the initial lipid composition, indicating no loss of a particular lipid during the preparation of extruded liposomes.

**Table 1-1 Lipid analysis of liposomes modified with aminoglycolipid.**

| Liposome | Lipid Composition (initial) (in molar ratio) | | | Lipid composition (after preparation) (in molar ratio) | | |
|---|---|---|---|---|---|---|
| | EPC | Chol | PGlcN | EPC | Chol | PGlcN |
| Control-L | 80 | 20 | - | 79.2 | 20.8 | - |
| PGlcN10-L | 70 | 20 | 10 | 70.5 | 19.9 | 9.6 |
| PGlcN20-L | 60 | 20 | 20 | 60.8 | 19.8 | 19.5 |
| PGlcN30-L | 50 | 20 | 30 | 50.9 | 19.2 | 29.9 |

*Characterization of liposomes*

The diameters of $VET_{200}$ studied here were about 200 nm with homogenous distribution as shown in Table 1-2. This indicated that basic lipids, PGlcN and SA did not influence the size of liposomes. Zeta potential values of liposomes composed of Egg PC, cholesterol and PGlcN or SA were positive in buffer solution at 25 °C, as shown in Table 1-2. The glucosamine group of PglcN has a positive charge due to the $+NH_3$ groups in the buffer solution (pH = 7.4), and incorporation of PGlcN into liposomal bilayers led to positive charges of the membranes. The difference of zeta potentials reflected the difference of pKa between PGlcN- and SA-liposomes. The pKa of

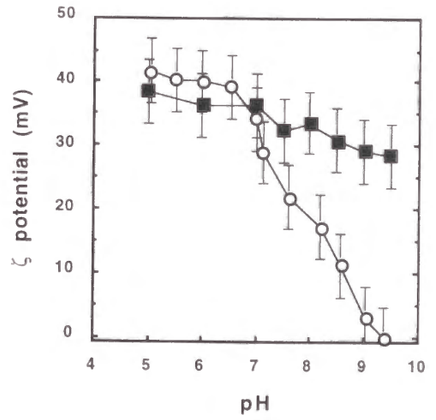


**Fig.1-1 ζ potential of liposomes prepared in various pH.**
Liposomes were prepared by extrusion at various pH.
(○), EPC:Chol:PGlcN (6:2:2 in molar ratio);
(■), EPC:Chol:SA (6:2:2 in molar ratio).

**Table 1-2 Diameter and zeta potential of liposomes prepared by extrusion method**

| Liposomal lipid composition (in molar ratio) | Diameter (nm) | Zeta potential (mV) |
|---|---|---|
| (in 10 mM Tris/300 mM Sorbitol, pH 7.4) | | |
| EPC:Chol (8:2) | 223.1 ± 24 | -3.94 ± 0.32 |
| EPC:Chol:PGlcN (7:2:1) | 204.5 ± 48 | 28.94 ± 0.26 |
| EPC:Chol:PGlcN (6:2:2) | 225.8 ± 25 | 41.12 ± 1.14 |
| EPC:Chol:PGlcN (5:2:3) | 232.3 ± 24 | 48.84 ± 0.46 |
| EPC:Chol:SA (7:2:1) | 236.8 ± 45 | 53.68 ± 0.67 |
| EPC:Chol:SA (6:2:2) | 202.6 ± 44 | 54.70 ± 1.14 |
| EPC:Chol:SA (5:2:3) | 213.2 ± 38 | 66.58 ± 0.44 |
| (in 10 mM Tris/150 mM NaCl, pH 7.4) | | |
| EPC:Chol:PGlcN (7:2:1) | 222.1 ± 31 | 6.59 ± 0.67 |
| EPC:Chol:PGlcN (6:2:2) | 215.6 ± 45 | 15.89 ± 0.32 |
| EPC:Chol:PGlcN (5:2:3) | 227.1 ± 28 | 23.34 ± 1.38 |
| EPC:Chol:SA (7:2:1) | 224.0 ± 31 | 13.00 ± 0.83 |
| EPC:Chol:SA (6:2:2) | 219.2 ± 25 | 31.40 ± 0.97 |
| EPC:Chol:SA (5:2:3) | 216.5 ± 27 | 42.92 ± 0.30 |

PGlcN-liposomes was approx. 7.7 and that of SA-liposomes was over 10, determined by the zeta potentials of liposomes prepared with buffer at varied pH as shown in Fig. 1-1. The zeta potentials of liposomes containing SA in 10 mM Tris buffer (pH 7.4) were higher than those in 10 mM Tris/150 mM NaCl (pH 7.4) buffer. This is due to the shielding of positive charge by the ionic atmosphere surrounding liposomes.

*Hemolytic activities and toxicity of cationic liposomes*

SA-liposomes showed hemolytic activity at a high lipid concentration range as shown in Fig. 1-2. On the other hand, there was no apparent hemolytic activity of conventional control liposomes and PGlcN-liposomes.

The 50 % lethal dose of SA-liposomes was about 1.5 mmol total lipid/kg in mice, while that of control liposomes and PGlcN-liposomes was much higher as shown in Fig. 1-3. The toxicity of PGlcN-liposomes needs further investigation, however, PGlcN-liposomes have the advantage of having low toxicity compared with SA-liposomes.

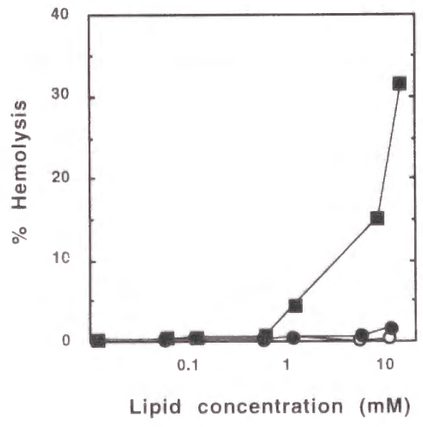


**Fig. 1-2 Hemolytic activity of liposomes.**
Liposomes were prepared by extrusion. Rat erythrocytes were incubated with a liposomal suspension for 1 hour. After centrifugation, the absorbance of the supernatants was measured. The values are expressed as a percentage of hemolysis.
(●), EPC:Chol (8:2 in molar ratio);
(○), EPC:Chol:PGlcN (6:2:2 in molar ratio);
(■), EPC:Chol:SA (6:2:2 in molar ratio).

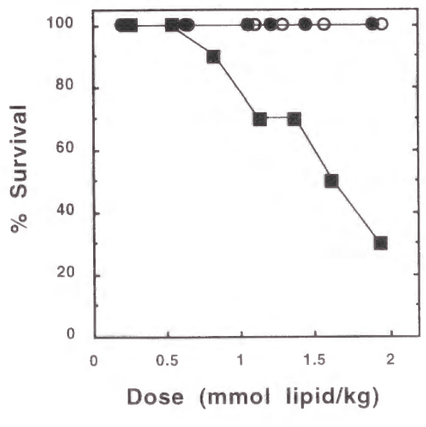


**Fig.1-3 The survival of mice treated with liposomes.**
Liposomes were prepared by extrusion and injected into tail vein of mice.
(●), EPC:Chol (8:2 in molar ratio);
(○), EPC:Chol:PGlcN (6:2:2 in molar ratio);
(■), EPC:Chol:SA (6:2:2 in molar ratio).



*Stability of liposomes in serum*

Table 1-4 shows the calcein retention in rat serum at 37 °C. About 90 % of calcein was retained in all liposomes after 8 hour incubation with rat serum. Incorporation of PGlcN into the egg PC/cholesterol bilayer did not cause an increase in calcein leakage from the liposomes in rat serum.

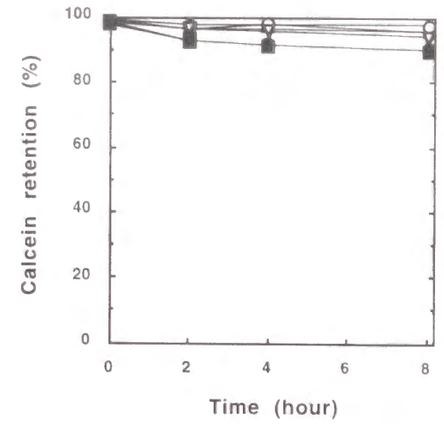


**Fig. 1-4 Stability of liposomes in rat serum.**
Liposomes with entrapped calcein were prepared by extrusion. Liposomal suspensions were incubated with rat serum at 37 °C, and retention of calcein in liposomes was measured fluorometrically.
(●), EPC:Chol (8:2 in molar ratio);
(▽), EPC:Chol:PGlcN (7:2:1 in molar ratio);
(○), EPC:Chol:PGlcN (6:2:2 in molar ratio);
(△), EPC:Chol:PGlcN (5:2:3 in molar ratio);
(▼), EPC:Chol:SA (7:2:1 in molar ratio);
(■), EPC:Chol:SA (6:2:2 in molar ratio).

*Entrapment efficiency of SOD in liposomes prepared by hydration*

The entrapment efficiency of SOD into liposomes prepared by various methods was determined by SOD nitrite assay, as shown in Table 1-3. The control liposomes composed of EPC and Chol (8:2 in molar ratio) could entrap about 1 % of SOD at 10 mM of lipid concentration in both buffers. The trapped volume of $VET_{200}$ was determined as 1.80 ± 0.18 l/mol (total lipid) by using calcein as an entrapped fluorescent marker. The entrapment efficiency of SOD in $VET_{200}$ is calculated as 1.8 % on the basis of the trap volume and lipid concentration of 10 mM. The experimental value determined by the nitrite method agreed roughly with the calculated value. Ten times the amount of SOD was entrapped into cationic liposomes composed of EPC, Chol and PGlcN (7:2:1 in molar ratio) in buffer of low ionic strength containing sorbitol, as compared with control liposomes by the hydration method. Cationic SA-liposomes also showed high entrapment efficiency when prepared in the buffer of low ionic strength. However, the entrapment efficiency of the PGlcN-liposomes in buffer of high ionic strength containing 150 mM NaCl, was only about 2 times higher than that of control liposomes. This is due to reduced electrostatic interaction between the negatively charged SOD and the positively charged lipid membrane, since the zeta poten-

tial of cationic liposomes in buffer containing 150 mM NaCl was lower than that in buffer containing sorbitol.

*Entrapment efficiency of SOD in liposomes prepared by reverse phase evaporation*

Reverse phase evaporation vesicles (REVs) have high entrapped aqueous volume per lipid [25]. Neutral REVs could entrap SOD about three times more than neutral extruded liposomes. The entrapment efficiency of cationic PGlcN-REVs in the buffer of low ionic strength determined by the nitrite method (SOD activity assay) was roughly similar to that of cationic PGlcN-extruded liposomes. The amount of entrapped SOD in PGlcN-REVs determined by the Lowry method was the highest, and almost 100 % of SOD was found in liposomes (data not shown). However, the amount of SOD in liposomes determined by the nitrite method (SOD activity assay) was much smaller (about 7 % as entrapment efficiency) than that determined by the Lowry method, indicating that more than 90 % of SOD entrapped into liposomes lost its enzymatic activity during preparation of REVs. Other methods did not inactivate SOD, because the activity of SOD in liposomal suspensions before gel filtration was the same as that in initial buffer. Therefore, the REV method is not suitable for entrapment of enzyme, since organic solvent and/or the sonication step in the preparation of liposomes probably inactivated the enzyme [43-45]. SOD alone in buffer was sonicated in order to confirm whether the sonication process inactivated SOD or not. The sonicated SOD retained its activity (data not shown) ; therefore, contact with organic solvent inactivated SOD. The problem remains that organic solvent may not be totally removed during reverse phase evaporation and cause discomfort during therapeutic applications.

**Table 1-3 The entrapment efficiency (%) of SOD into liposomes determined by SOD activity assay.**

| liposomal lipid composition (in molar ratio) | buffer medium | Method of vesicle preparation: Hydration (Extrusion) | Reverse phase evaporation | Freeze-thaw | Dehydration-rehydration |
|---|---|---|---|---|---|
| EPC:Chol (8:2) | 10 mM Tris/150 mM NaCl | 0.9 ± 0.1 | 2.4 ± 0.1 | 3.2 ± 0.1 | 1.1 ± 0.1 |
| | 10 mM Tris/300 mM sorbitol | 0.7 ± 0.4 | 2.9 ± 1.1 | 0.9 ± 0.0 | 0.7 ± 0.1 |
| EPC:Chol:PGlcN (7:2:1) | 10 mM Tris/150 mM NaCl | 1.7 ± 0.2 | 2.8 ± 0.4 | 2.8 ± 0.1 | 0.8 ± 0.4 |
| | 10 mM Tris/300 mM sorbitol | 8.5 ± 1.0 | 7.1 ± 4.6 | 6.8 ± 0.0 | 10.9 ± 0.6 |
| EPC:Chol:SA (7:2:1) | 10 mM Tris/150 mM NaCl | 1.3 ± 0.1 | 2.9 ± 0.3 | 3.6 ± 0.1 | 2.7 ± 0.3 |
| | 10 mM Tris/300 mM sorbitol | 21.3 ± 1.2 | 32.0 ± 2.8 | 12.8 ± 3.6 | 21.9 ± 0.9 |

Liposomes were prepared by various methods at a total lipid concentration of 10 mM as described in the text. Amount of SOD in liposomes was determined by the Nitrite method after removal of untrapped SOD by gel filtration assuming no loss of activity. Values are expressed as the mean ± S.D. (n = 3).



Cationic SA-REVs in the buffer of low-ionic strength were also able to entrap large amounts of SOD, about 30 % as determined by the nitrite method and 100 % as determined by the Lowry method (data not shown). In this case, 70 % of the SODs was inactivated during liposome preparation. The different levels of SOD inactivation between PGlcN-liposomes and SA-liposomes can be explained as follows: The latter had a larger positive charge than the former and immediately trapped SOD with shorter contact time with organic solvent.

*Entrapment efficiency of SOD in liposomes prepared by freeze-thawing*

At high ionic strength, the freeze-thawing method gave three times larger trapping efficiency of SOD than the extrusion method in neutral liposomes. In contrast, at low ionic strength, both methods showed similar efficiencies as shown in Table 1-3. Table 1-4 shows the diameters of the liposomes before and after freeze-thawing without extrusion. In a buffer containing NaCl, liposomes fused and effectively encapsulated SODs during freeze-thawing. On the other hand, in the presence of sorbitol, the

**Table 1-4 Diameters of liposomes before and after freeze-thaw and dehydration-rehydration**

| Method | Liposomes | EPC:Chol (8:2) | | EPC:Chol:PGlcN (7:2:1) | | EPC:Chol:SA (7:2:1) | |
|---|---|---|---|---|---|---|---|
| | Buffer medium | before | after | before | after | before | after |
| Freeze-thaw | 150mM NaCl | 237.2 ± 36 | 555.9 ± 319 | 210.9 ± 32 | 185.7 ± 87 | 226.9 ± 36 | 175.8 ± 52 |
| | 300mM sorbitol | 228.8 ± 32 | 233.8 ± 45 | 221.4 ± 36 | 170.4 ± 157 | 226.4 ± 32 | 128.3 ± 54 |
| Dehydration | 150mM NaCl | 219.1 ± 19 | 893.0 ± 93 | 206.3 ± 20 | 877.0 ± 109 | 211.3 ± 32 | 862.3 ± 234 |
| -rehydration | 300mM sorbitol | 233.1 ± 24 | 460.1 ± 126 | 204.5 ± 48 | 375.3 ± 100 | 210.8 ± 37 | 488.8 ± 154 |

Liposomes were initially prepared by the extrusion method ($VET_{200}$).
Liposome size after freeze-thawing or dehydration-rehydration was measured before extrusion.
Values are expressed as the mean diameter ± S.D. (nm).

neutral liposomes did not collapse during freeze-thawing, because sorbitol prevented membrane fusion of liposomes [46, 47]. Therefore, SOD was not entrapped into liposomes in sorbitol buffer by the freeze-thawing method as shown in Fig. 1-3.

In the case of cationic PGlcN- or SA-liposomes in sorbitol buffer, a large amount of SOD was entrapped into liposomes. The diameter of cationic liposomes decreased after freeze-thawing. Westman et al. reported that freeze-thawing reconstructs liposomes which are smaller in size than liposomes that had been only vortexed [48]. The decrease of liposome size indicated that liposomal membranes are disrupted by ice in the frozen state and form fragmented lipid assemblies. These assemblies are reconstituted to liposomes during thawing. SODs could be effectively entrapped into cationic liposomes through electrostatic attraction during the reconstitution of liposomes. The electrostatic repulsion between fragmented lipid assemblies would induce the formation of smaller liposomes before freezing.

*Entrapment efficiency of SOD in liposomes prepared by dehydration-rehydration*

The entrapment efficiency of SOD by the rehydration-dehydration method was similar to that by hydration as shown in Fig. 1-3. Cationic liposomes in sorbitol buffer entrapped a large amount of SOD by the electrostatic interaction. Table 1-4 shows that the diameter of liposomes increased after dehydration-rehydration. This indicats that the bilayer structure of liposomes after freeze-drying does not remain intact. The liposomes (closed vesicles) change to open fragments of bilayers during freeze-drying, and entrap the SOD in the reconstruction during rehydration [49, 50].

*Effect of amount of incorporated basic lipid*

From the findings described above, it was clear that hydration followed by extrusion in a buffer of low ionic strength was simple and entrapped a large amount of SOD. Thus, we investigated the effect of the amount of incorporated basic lipids on the trapping efficiency. Fig. 1-5 shows the entrapment efficiency of SOD by hydration method as a function of mol% of basic lipid (PGlcN or SA) in liposomes prepared in



NaCl or sorbitol buffer. In the buffer of low ionic strength, the entrapment of SOD into cationic liposomes markedly increased with the basic lipid content. The hydration method using high mol% of basic lipid with buffer of low ionic strength, was most effective for entrapment of enzyme without loss of activity. Entrapment efficiency of SOD into PGlcN- or SA-liposomes in buffer of high ionic strength was low due to decreased electrostatic interaction of lipid bilayer and SOD.

Fig. 1-6 shows the entrapment efficiency of SOD versus the zeta potential of liposomes. The entrapment in buffer of low ionic strength increased with the zeta potential of liposomes. Despite high zeta potential of SA-liposomes (i.e. EPC:Chol:SA = 6:2:2 or 5:2:3), the entrapment efficiency was lower in NaCl buffer than that of PGlcN-liposomes (EPC:Chol:PGlcN = 7:2:1 or 6:2:2) in sorbitol buffer. These results indicate that electrostatic interaction is crucial for effective entrapment.

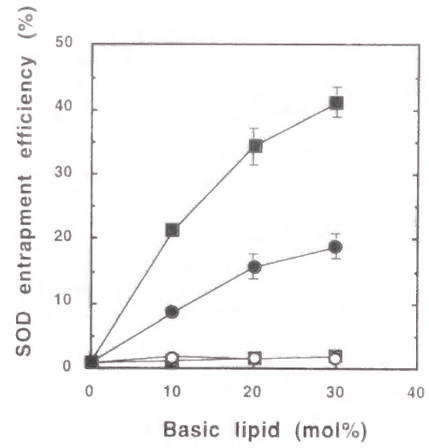


**Fig. 1-5 The trapping efficiency of SOD into liposomes**

The trapping efficiency of SOD into liposomes determined by the nitrite method as a function of the mol % of basic lipid. Liposomes were prepared by extrusion at 10 mM lipid concentration as described in the text.
(▽) liposomes (EPC:Chol=8:2 in molar ratio) in 10 mM Tris/300 mM sorbitol (pH 7.4); (●) PGlcN-liposomes in 10 mM Tris/300 mM sorbitol (pH 7.4); (○) PGlcN-liposomes in 10 mM Tris/150 mM NaCl (pH 7.4); (■) SA-liposomes in 10 mM Tris/300 mM sorbitol (pH 7.4); (□) SA-liposomes in 10 mM Tris/150 mM NaCl (pH 7.4).

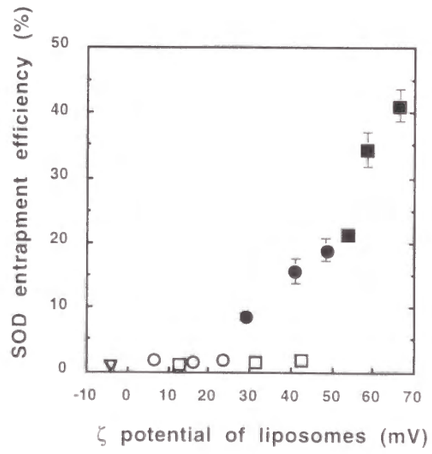


**Fig. 1-6 Zeta potential versus SOD entrapment.**

(▽) liposomes (EPC:Chol=8:2 in molar ratio) in 10 mM Tris/300 mM sorbitol (pH 7.4); (●) PGlcN-liposomes in 10 mM Tris/300 mM sorbitol (pH 7.4); (○) PGlcN-liposomes in 10 mM Tris/150 mM NaCl (pH 7.4); (■) SA-liposomes in 10 mM Tris/300 mM sorbitol (pH 7.4); (□) SA-liposomes in 10 mM Tris/150 mM NaCl (pH 7.4).

### Conclusion

The author synthesized amino-glycolipid, PGlcN as basic lipid. This newly synthesized PGlcN distributed in EPC/Chol bilayers stably, gave positive charge to liposomes, and showed low toxicity compared with stearylamine. The author demonstrated that liposomes containing basic lipids entrapped a large amount of negatively charged substances (SODs) by hydration and dehydration-rehydration methods without loss of activity. The entrapment efficiency increased with increase of basic lipid concentration in liposomal membrane. These methods based on the electrostatic interaction can effectively and simply entrap anionic compounds that cannot be entrapped by reverse phase or pH-gradient methods.



# Chapter 2

## Disposition kinetics of cationic liposomes in rats

It is desirable to achieve a reasonable concentration of the drugs at the target site and low concentration at other sites to prevent side effects. Liposomes have attracted large attention due to their various advantages as drug carriers, being biodegradable, having low toxicity and being able to encapsulate hydrophilic, lipophilic and amphiphilic drugs. However, there are inevitable drawbacks in the use *in vivo*. The utilization of liposomes as drug carriers is hampered by rapid clearance by the reticuloendothelial system (RES). Their rapid removal from the circulation following intravenous administration is primarily due to phagocytosis by the Kupffer cells and macrophages of the spleen [4, 5]. The reason for the efficient uptake of injected liposomes by RES in not currently well understood, but is thought to be related to the opsonin of liposomes by plasma proteins [51-56]. Native liposomes are especially suitable for targeting diseases of RES such as leishmaniasis and fungal infections [6, 7] but are undesirable for delivering drugs to other organs.

Numerous attempts have been made to prolong liposome lifetime in the circulation, using such methods as incorporation of cholesterol (Chol) [9], RES blockade by presaturation with 'empty' liposomes [57, 58], and coating with ganglioside $G_{M1}$ [10] or polyethylene glycol (PEG) [11-14]. Cationic stearylamine(SA)-liposomes also remain in the blood longer than neutral or anionic liposomes (containing phosphatidylserine or phosphatidic acid) [15]. Cationic liposomes have been also studied as tools for the delivery of plasmid DNA and RNA into cells [16-18]. However, SA is difficult to apply therapeutically because of its toxicity [19, 21].

In this chapter, the author synthesized several biodegradable monoesters of fatty acid with amino sugars or neutral sugars and prepared liposomes modified with these glycolipids. The effects of the positive charge, sugar structure, and the charge density on the disposition property of liposomes in rat were investigated.

## 2-1 Materials and Methods

*Materials*

Egg yolk L-α-phosphatidylcholine (EPC), stearylamine (SA) and calcein were obtained as described in 1-1. Lactosylceramide (LacCer) was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [1,2(n)-$^3$H]-cholesteryl hexadecyl ether, $^3$H-CHE was purchased from Daiichi Pure Chemical Co. Ltd. (Tokyo, Japan). Soluene-350® was purchased from Packard Instrument Co. Inc. (U.S.A.). Clear-sol I was obtained from Nacalai Tesque Inc. (Kyoto, Japan). All other chemicals from Wako Pure Chemical Ind. Ltd. (Osaka, Japan) were of special grade. Water was glass distilled twice.

*Synthesis of methyl-2-amino 6-palmitoyl-D-glucoside*

Methyl-2-amino-2-deoxy-6-*O*-palmitoyl-D-glucoside (PGlcN), methyl-2-amino-2-deoxy-6-*O*-palmitoyl-D-galactoside (PGalN), methyl-2-amino-2-deoxy-6-*O*-palmitoyl-D-mannoside (PManN), methyl-6-*O*-palmitoyl-D-glucopyranoside (PGlc), methyl-6-*O*-palmitoyl-D-galactopyranoside (PGal), and methyl-6-*O*-palmitoyl-D-mannopyranoside (PMan) (Fig. 2-1) were synthesized in the manner described in Chapter 1.

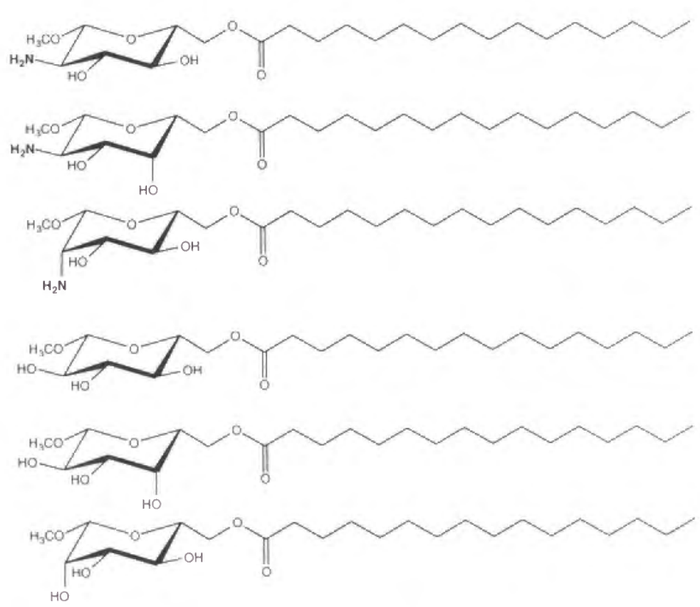

**Fig. 2-1**

Structure of methyl-2-amino-2-deoxy-6-*O*-palmitoyl-D-glucoside (PGlcN), methyl-2-amino-2-deoxy-6-*O*-palmitoyl-D-galactoside (PGalN), methyl-2-amino-2-deoxy-6-*O*-palmitoyl-D-mannoside (PMan), methyl-6-*O*-palmitoyl-D-glucopyranoside (PGlc), methyl-6-*O*-palmitoyl-D-galactopyranoside (PGal), and methyl-6-*O*-palmitoyl-D-mannopyranoside (PMan).



*Preparation of liposomes*

Multilamellar vesicles (MLVs) were prepared by extrusion [40]. Liposomes were composed of egg phosphatidylcholine, cholesterol and glycolipid in the desired molar ratios. To prepare lipid labeled liposomes, $^3$H-CHE as a non-exchangeable and non-degradable marker [59] was added to the lipid mixture. The thin lipid film was dried overnight *in vacuum*, then hydrated with phosphate buffered saline (8.1 mM $Na_2HPO_4$/ 1.47 mM $KH_2PO_4$/137.9 mM NaCl/2.7 mM KCl, PBS, pH 7.4). The suspensions were successively extruded through polycarbonate filters of various pore sizes (0.6 and 0.2 μm 5 times, $VET_{200}$: vesicles with a diameter of 200 nm prepared by an extrusion technique). The sizes of the liposomes were measured by dynamic light scattering on a Photal laser particle analyzer (LPA-3100, Otsuka Electronics Co. Ltd., Japan) connected to a photon correlater (LPA-3000). Zeta potential of liposomes with a diameter of about 200 nm was calculated by Smoluchowski's equation [42] from their electrophoretic mobilities in PBS (pH 7.4) at 25 °C. The mobilities were obtained from electrophoretic light scattering measurements (Zetasizer 4, Malvern Instruments, U. K.).

*Stability of liposomes in serum*

The thin lipid films were hydrated with 1 mM calcein/10 mM Tris/150 mM NaCl buffer (pH 7.4). The suspensions were vortexed and extruded through polycarbonate filters as described above. Untrapped calcein was removed by gel-filtration (Bio-Gel A-1.5m, 10 mM Tris/150 mM NaCl buffer (pH 7.4) as an elute). One milliliter of the liposomal suspension was mixed with 4 ml of prewarmed rat serum and the mixture was incubated at 37 °C. The retention of calcein in liposomes was determined fluorometrically (excitation at 490 nm and emission at 520 nm) (RF-5000, Shimadzu Co., Japan). The percentage of retention was calculated from Eqn 1 described in 1-1.

*Studies in vivo*

Liposomes labeled with $^3$H-CHE (15 μmol of total lipid per kg) were injected into the femoral vein of three Nembutal-anesthetized male Wistar rats, weighing from 180

to 200 g. At regular intervals, blood samples were collected from the jugular vein with a heparinized syringe and centrifuged at 2000 x g for 2 minutes to obtain the plasma. To determine the accumulated amounts of the liposomes in each organ at 8 hours after injection, the liver was perfused via the portal vein with isotonic saline to remove the blood. Thereafter the liver, spleen, kidney, heart, lung, intestine, muscle and lymph were collected and washed with saline. Around 50 mg of tissue was dissolved in Soluene-350, neutralized with HCl, then Clear-sol I was added. The radioactivity of samples was counted on a scintillation counter (LS5000TA, Beckman, U.S.A.). The tissue samples were examined in triplicate.

The plasma liposome concentrations over time data after the intravenous injection showed a bi-exponential decay profile. The time course of the concentration of lipid in plasma was therefore numerically fitted to a two-compartment model using the fitting program MULTI [60]. A simple analysis variance test method (*t*-test) was employed to assess the significance of the observed differences in the pharmacokinetics following intravenous injection of liposomes.

*Lectin-induced aggregation*

*Momordica charantia* lectin was added rapidly to a liposomal suspension, in 10 mM Tris/150 mM NaCl (pH 7.4) buffer, the aggregation was followed by the turbidity increase with time at 540 nm on a spectrophotometer (UV-265FW, Shimadzu Co., Japan) at 25 °C [61]. The incubation mixtures contained liposomes at a total lipid concentration of 0.5 mM and 50 μg of lectin in a total volume of 2.1 ml.

*Quantification of the amount of total protein associated with liposomes*

Liposomes were retrieved from liposome-plasma mixture using a spin column according to the procedure described by Chonn et al. [62]. Briefly, Bio-Gel A-15m, 200-400 mesh (Bio-Rad, Richmond, CA, USA) was equilibrated with 10 mM HEPES/ 150 mM NaCl (pH 7.4) buffer and packed in 1.0 ml Tuberculin syringes with glass wool plugs. Liposomes labeled with 1,1'-dioctadecyl-3,3,3',3'-



tetramethylindocarbocyanine perchlorate (DiI; Fluoreszenztechnologie, Graz, Austria) was prepared by extrusion in 10 mM HEPES/150 mM NaCl (pH 7.4) buffer. Liposomes labeled with DiI (100 μmol of total lipid per kg) were injected into the femoral vein of three Nembutal-anesthetized male Wistar rats. After 5 minutes, blood samples were collected from the jugular vein with a heparinized syringe and centrifuged at 2000 x g for 2 minutes to obtain the plasma. Aliquots of the liposome-plasma mixtures (75 μl) were then applied to spin columns and immediately centrifuged (1000 rpm x 1 min). Column fractions were collected in glass culture tubes by applying 75 μl of 10 mM HEPES/150 mM NaCl (pH 7.4) buffer to the spin columns and centrifuging (1000 rpm x 1 min). The elute from each centrifugation step was considered to be one fraction. Liposomes eluted in fraction 4-6 were pooled for subsequent experiments. Complete separation of liposomes from free plasma proteins was confirmed by separate elution of liposomes and rat plasma.

Liposome-associated proteins were extracted and delipidated according to the procedure described by Wessel and Flugge [63]. Protein was quantified using the Micro BCA Protein Assay Reagent Kit (Pierce Chemical, Rockford, IL, USA) . Briefly, 150 μl 0.2 % SDS-0.02 N NaOH protein solution was added to 150 μl of protein assay working reagent, and then incubated at 37 °C for 3 hours. Absorbance of sample was compared to a bovine serum albumin standard curve at 562 nm [64]. The amount of bound protein (g protein/ mol total lipid) was calculated on the basis of the lipid concentrations of the recovered liposomes determined by measurement of fluorescence intensity of DiI.

*SDS-polyacrylamide gel electrophoretic analysis*

Protein separation was performed by SDS-PAGE using AE-6400 Rapidas mini slab electrophoretic apparatus (ATTO, Tokyo, Japan) on precast 5-20 % gradient PAGEL gels (ATTO) under non-reducing conditions. The gels were stained with Coomassie Blue to visualize the proteins.

## 2-2 Results

*Properties of liposomes*

Table 2-1 shows the average diameter and zeta potential of the liposomes modified with glycolipids studied in this chapter. Based on dynamic light scattering, the mean diameters of liposomes were about 200 nm with homogeneous distribution, indicating that glycolipid did not influence the size of liposomes. Electron micrographs revealed that the liposomes were multilamellar. The zeta potentials of control and neutral sugar coated-liposomes were about -4 mV. Zeta potential values of liposomes containing aminoglycolipids were positive.

About 90 % of calcein was retained in liposomes modified with glycolipids after an 8 hour incubation with rat serum as shown in Table 2-1. Incorporation of glycolipids into the egg PC/cholesterol bilayer did not cause leakage of the dye out of liposomes in rat serum.

*Effect of glycolipid on the clearance of liposomes*

As shown in Fig. 2-2, the radioactivity of cationic PGlcN-, PGalN- or PManN-L was larger than that of control liposomes in the plasma at every point determined. The estimated AUC and MRT values of PGlcN-, PGalN- or PManN-L were twice those of control liposomes. The uptake of PGlcN-, PGalN- or PManN-L by the liver at 8 hours after i.v. administration decreased compared with that of Control-L as shown in Fig. 2-3. However, the uptake of PGlcN-, PGalN- or PManN-L in the spleen at 8 hours after i.v. administration was comparable to that of Control-L. There is no significant difference in

**Table 2-1 The physico-chemical properties of liposomes.**

| Liposome | Lipid Composition (in molar ratio) | Diameter (nm) | Zeta potential (mV) | Retention[1)] (%) |
|---|---|---|---|---|
| Control-L | EPC:Chol=6:2 | 236.8 ± 33 | -4.3 ± 0.6 | 90.2 |
| PGlcN-L | EPC:Chol:PGlcN=6:2:2 | 225.8 ± 25 | 15.9 ± 0.3 | 90.7 |
| PGalN-L | EPC:Chol:PGalN=6:2:2 | 237.6 ± 44 | 18.8 ± 0.6 | 89.1 |
| PManN-L | EPC:Chol:PManN=6:2:2 | 243.8 ± 49 | 13.0 ± 0.7 | 89.3 |
| PGlc-L | EPC:Chol:PGlc=6:2:2 | 197.5 ± 21 | -3.9 ± 0.6 | 88.1 |
| PGal-L | EPC:Chol:PGal=6:2:2 | 217.1 ± 21 | -4.7 ± 1.2 | 87.0 |
| PMan-L | EPC:Chol:PMan=6:2:2 | 238.7 ± 24 | -3.7 ± 0.6 | 87.4 |

1) The calcein retention in liposomes was determined fluorometrically after incubation with rat serum for 8 hours at 37 °C



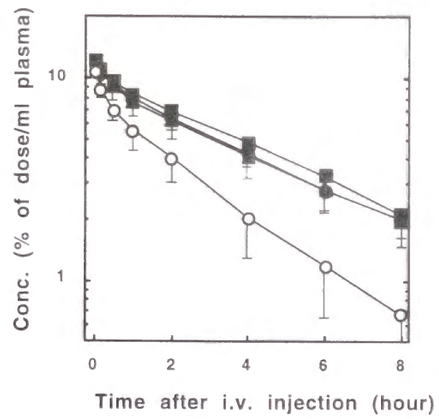


**Fig. 2-2**

Blood clearance of cationic liposomes modified with aminoglycolipids in the rat after an intravenous injection. The liposomes labeled with $^{3}$H-CHE were prepared by extrusion ($VET_{200}$) and injected into the femoral vein of rats at a dose of 15 μmol of total lipid/kg. Each value is expressed as a percentage ± S.D. of the administered $^{3}$H-CHE radioactivity per ml of plasma. n = 3.
(○),EPC:Chol (8:2 in molar ratio);
(●), EPC:Chol:PGlcN (6:2:2 in molar ratio);
(■), EPC:Chol:PGalN (6:2:2 in molar ratio);
(▲), EPC:Chol:PManN (6:2:2 in molar ratio).

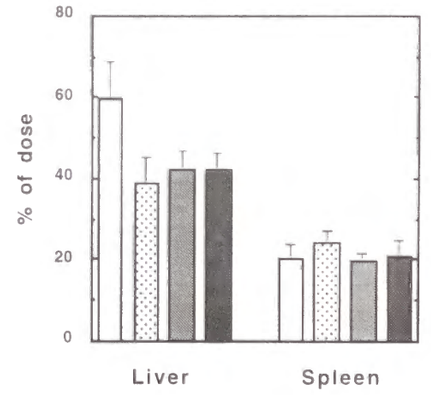


**Fig. 2-3**

Tissue distribution of cationic liposomes modified with aminoglycolipids in the rat 8 hr after an intravenous injection. The liposomes labeled with $^{3}$H-CHE were prepared by extrusion ($VET_{200}$) and injected into the femoral vein of rats at a dose of 15 μmol of total lipid/kg. Each value is expressed as a percentage ± S.D. of the administered $^{3}$H-CHE radioactivity per total organ. n = 3.
(▭), EPC:Chol (8:2 in molar ratio);
(▭), EPC:Chol:PGlcN (6:2:2 in molar ratio);
(▭), EPC:Chol:PGalN (6:2:2 in molar ratio);
(▭), EPC:Chol:PManN (6:2:2 in molar ratio).

*in vivo* behavior among the three cationic liposomes modified with aminoglycolipids.

In contrast to the cationic liposomes, the disposition characteristics of the neutral sugar-modified PGlc-, PGal- or PMan-L were almost the same as that of Control-L as shown in Fig. 2-4. The uptake in the liver and spleen of PGlc-, PGal- or PMan-L was not different from that of Control-L 8 hours after i.v. injection (data not shown).

*Lectin-induced aggregation*

The increase of turbidity was observed by the addition of *Momordica charantia* lectin to a suspension of LacCer-liposomes (incorporation of 20 mol % lactosylceramide), while no aggregation was observed by the same treatment to PGalN- and PGal-liposomes as shown in Fig. 2-5.

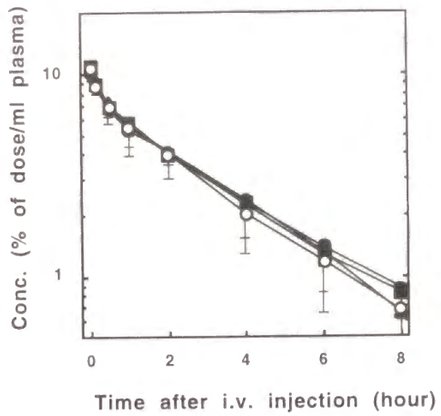


**Fig 2-4**

Blood clearance of liposomes modified with neutral-glycolipids in the rat after an intravenous injection. The liposomes labeled with $^3$H-CHE were prepared by extrusion ($VET_{200}$) and injected into the femoral vein of rats at a dose of 15 μmol of total lipid/kg. Each value is expressed as a percentage ± S.D. of the administered $^3$H-CHE radioactivity per ml of plasma. n = 3.
(○), EPC:Chol (8:2 in molar ratio);
(●), EPC:Chol:PGlc (6:2:2 in molar ratio);
(■), EPC:Chol:PGal (6:2:2 in molar ratio);
(▲), EPC:Chol:PMan (6:2:2 in molar ratio).

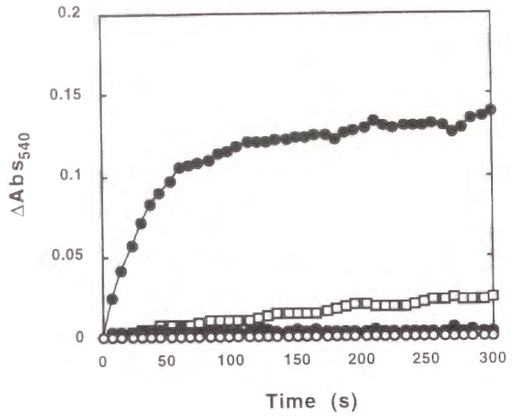


**Fig. 2-5**

Lectin induced aggregation followed by turbidity.
(○), EPC:Chol (8:2 in molar ratio);
(●), EPC:Chol:PGalN (6:2:2 in molar ratio);
(□), EPC:Chol:PGal (6:2:2 in molar ratio);
(●), EPC:Chol:LacCer (6:2:2 in molar ratio)

*Effect of positive charge density on the liposomal surface on the clearance of liposomes*

Next, the effect of the positive charge density on the liposomal surface on the disposition kinetics in rat was investigated. Table 2-2 shows the diameter and zeta potential of liposomes. The radioactivity of PGlcN-liposomes in rat plasma after intravenous injection is shown in Fig. 2-6 The radioactivity of the liposomes containing 10 mol% of PGlcN (PGlcN10-L) was slightly larger than that of the controls. The hepatic uptake of PGlcN10-L at 8 hours after the intravenous injection

**Table 2-2 The physico-chemical properties of liposomes.**

| Liposome | Lipid Composition (in molar ratio) | | Diameter (nm) | Zeta potential (mV) |
|---|---|---|---|---|
| Control-L | EPC:Chol | (8:2) | 236.8 ± 33 | -4.3 ± 0.6 |
| PGlcN10-L | EPC:Chol:PGlcN | (7:2:1) | 212.6 ± 32 | 6.6 ± 0.7 |
| PGlcN20-L | EPC:Chol:PGlcN | (6:2:2) | 225.8 ± 25 | 15.9 ± 0.3 |
| PGlcN30-L | EPC:Chol:PGlcN | (5:2:3) | 232.3 ± 24 | 23.3 ± 1.4 |
| PGlcN40-L | EPC:Chol:PGlcN | (4:2:4) | 219.1 ± 18 | 28.4 ± 1.1 |
| SA5-L | EPC:Chol:SA | (7.5:2:0.5) | 214.5 ± 35 | 8.0 ± 0.7 |
| SA10-L | EPC:Chol:SA | (7:2:1) | 236.8 ± 45 | 15.5 ± 1.0 |
| SA15-L | EPC:Chol:SA | (6.5:2:1.5) | 236.5 ± 25 | 26.7 ± 0.8 |
| SA20-L | EPC:Chol:SA | (6:2:2) | 202.6 ± 44 | 32.1 ± 0.5 |



was not significantly different from that of Control-L as shown in Fig. 2-7 ($p>0.05$). The radioactivity of the liposomes containing 20 (PGlcN20-L) or 30 (PGlcN30-L) mol% of PGlcN in the plasma was higher than that of Control-L at every point determined (Fig. 2-6). The uptakes of PGlcN20-L and PGlcN30-L in the liver were decreased compared with that of the controls (Fig. 2-7). The radioactivity of the liposomes containing 40 mol% of PGlcN (PGlcN40-L) was rapidly eliminated from the plasma within 1 hour after the injection, and thereafter decreased slowly in the plasma. The uptake of PGlcN40-L by the liver was slightly higher than that of Control-L.

In the case of stearylamine, the radioactivity of the liposomes containing 5 mol% of SA (SA5-L) in the plasma was slightly higher than that of Control-L as shown in Fig. 2-8. The radioactivity of the liposomes containing 10 mol% of SA (SA10-L) in the plasma was appreciably higher than that of Control-L, and the hepatic uptake of SA10-L decreased as shown in Fig. 2-9. However, the radioactivity of the liposomes containing 15 (SA15-L) or 20 (SA20-L) mol% of stearylamine in the plasma after the injection was less than that of Control-L, and the hepatic uptakes of SA15-L and SA20-L were

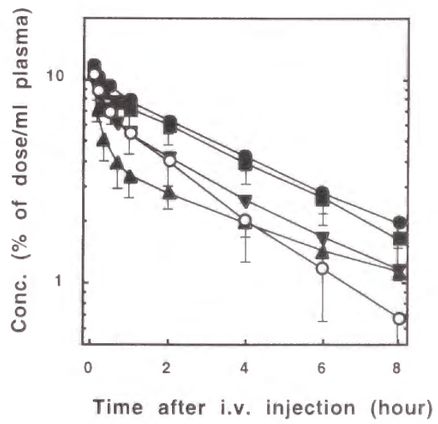


**Fig. 2-6**

Blood clearance of PGlcN-liposomes in the rat after an intravenous injection. The liposomes labeled with $^3$H-CHE were prepared by extrusion ($VET_{200}$) and injected into the femoral vein of rats at a dose of 15 μmol of total lipid/kg. Each value is expressed as a percentage ± S.D. of the administered $^3$H-CHE radioactivity per ml of plasma. n = 3.
(○), Control-L; (▼), PGlcN10-L; (●), PGlcN20-L; (■), PGlcN30-L; (▲), PGlcN40-L

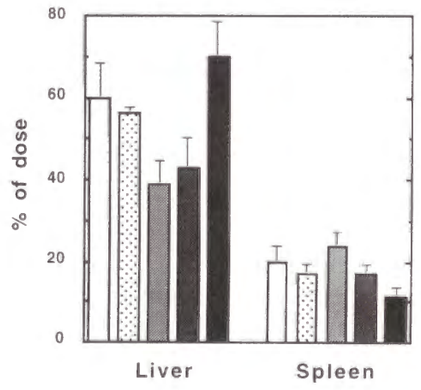


**Fig. 2-7**

Tissue distribution of PGlcN-liposomes in the rat 8 hr after an intravenous injection. The liposomes labeled with $^3$H-CHE were prepared by extrusion ($VET_{200}$) and injected into the femoral vein of rats at a dose of 15 μmol of total lipid/kg. Each value is expressed as a percentage ± S.D. of the administered $^3$H-CHE radioactivity per total organ. n = 3.
(▭), Control-L; (▭), PGlcN10-L; (▭), PGlcN20-L; (▭), PGlcN30-L; (▭), PGlcN40-L

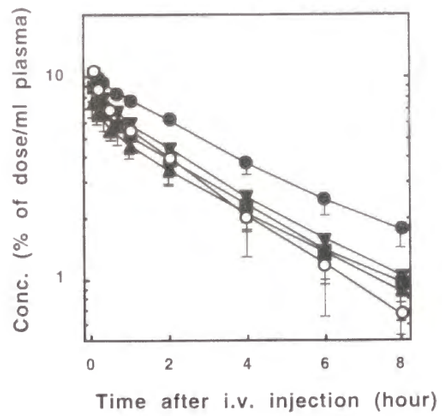


**Fig. 2-8**
Blood clearance of SA-liposomes in the rat after an intravenous injection. The liposomes labeled with $^3$H-CHE were prepared by extrusion ($VET_{200}$) and injected into the femoral vein of rats at a dose of 15 μmol of total lipid/kg. Each value is expressed as a percentage ± S.D. of the administered $^3$H-CHE radioactivity per ml of plasma. n = 3.
(○), Control-L; (▼), SA5-L; (●),SA10-L; (■),SA15-L; (▲),SA20-L

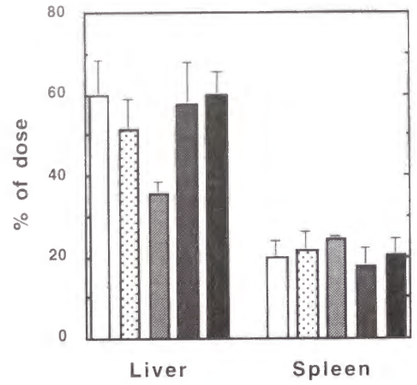


**Fig. 2-9**
Tissue distribution of SA-liposomes in the rat 8 hr after an intravenous injection. The liposomes labeled with $^3$H-CHE were prepared by extrusion ($VET_{200}$) and injected into the femoral vein of rats at a dose of 15 μmol of total lipid/kg. Each value is expressed as a percentage ± S.D. of the administered $^3$H-CHE radioactivity per total organ. n = 3.
(▭), Control-L; (▭), SA5-L; (▭), SA10-L; (▭), SA15-L; (▭), SA20-L

almost the same as that of Control-L.

Fig. 2-10 shows the zeta potentials of liposomes versus the area under the curve

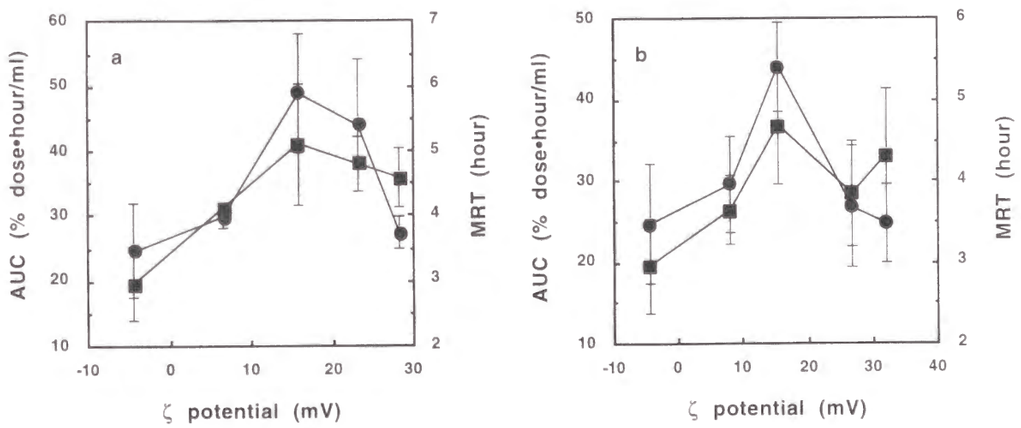


**Fig. 2-10**
Pharmacokinetic parameters as a function of zeta potential of liposomes. The values were obtained from the fitting program MULTI and are expressed as means ± S.D.. n=3.
(a) PGlcN-liposomes; (b) SA-liposomes; (circle), area under the curve, AUC; (square), mean residence time, MRT



(AUC) and mean residence time (MRT) obtained from a fitting program (MULTI). The estimated AUCs and MRTs of PGlcN10-L and SA5-L were not significantly different from those of Control-L ($p>0.05$). The AUCs and MRTs of PGlcN20-L, PGlcN30-L and SA10-L were about twice as large as those of Control-L. The AUCs of PGlcN40-L, SA15-L and SA20-L were comparable to that of Control-L, although their MRTs were larger than that of Control-L. The maximum points in AUC and MRT were obtained at about 15 mV regardless of lipid (PGlcN or SA). Thus, the AUC and MRT of cationic liposomes with zeta potentials of about +15 mV were largest, and the uptakes of these liposomes in the liver were the most strongly suppressed.

*Plasma protein associated with liposomes*

The amount of rat plasma proteins associated with liposomes was almost the same among the liposomes studied here as shown in Fig. 2-11. SDS-PAGE analysis revealed no obvious difference of protein profiles for different compositions of liposomes as shown in Fig. 2-12.

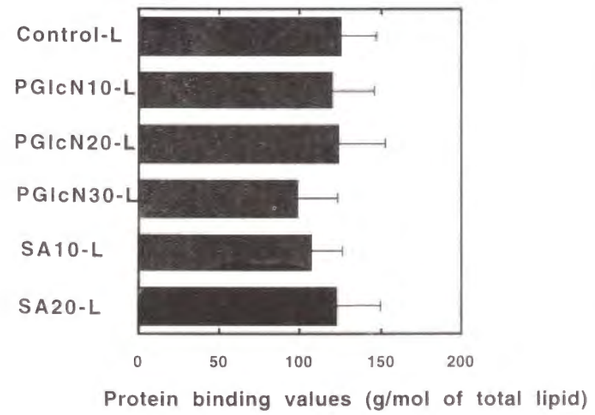


**Fig. 2-11**
The amount of plasma protein associated with liposomes. Liposomes labeled with DiI were injected into rats and liposomes-plasma mixtures were obtained. Liposomes were retrieved from the liposome-plasma mixtures using a spin column and protein was quantified using the Micro BCA Protein Assay.

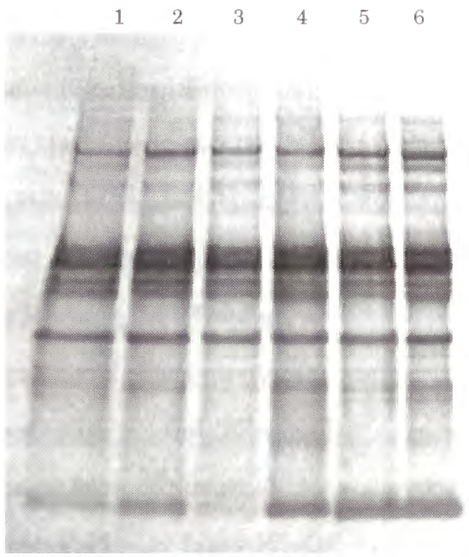


**Fig. 2-12**
SDS-profile of the protein associated with liposomes. The proteins associated with liposomes recovered liposome-serum mixture using spin column were separated on a 5-20 % SDS-PAGE gel under non-reducing conditions.
Lane 1, Control-L; Lane 2, PGlcN10-L;
Lane 3, PGlcN20-L; Lane 4, PGclN30-L;
Lane 5, SA10-L; Lane 6, SA20-L

## 2-3 Discussion

*Effect of glycolipid on the clearance of liposomes*

Cationic liposomes containing 20 mol% of aminoglycolipids had a longer half-life in the bloodstream than control liposomes and were less accumulated in the liver after an intravenous injection. It is predicted that the suppression of the hepatic uptake of these liposomes prolonged the circulation time. On the other hand, the uptake in the spleen of cationic liposomes modified with amino sugar was not different from that of the control liposomes. The spillover phenomenon may lead to no decrease of the cationic liposomal uptake by the spleen [58]. The clearance rate and the tissue distribution of neutral sugar-modified liposomes were almost the same as that of control liposomes. Allen *et al.* showed that glucosylceramide, monoglucosyldiacylglycerol and galactosylceramide were also incapable of decreasing the uptake of the liposomes by RES [65]. These findings suggest that the positive charge but not sugar structure on aminoglycolipids is an important factor in avoiding RES.

The disposition kinetics of liposomes depend on the size of liposomes. Small liposomes of diameters less than 100 nm can pass through the fenestration in the sinusoids or through the region of increased capillary permeability and gain access to liver parenchymal cells [66]. Multilamellar vesicles with a diameter of 200 nm are mainly taken up by the Kupffer cells in the liver.

The parenchymal cells have a galactose specific receptor and the nonparenchymal cells (Kupffer cells) have a mannose one [67]. Consequently, liposomes containing lactosylceramide were reported to accumulate in the liver using a galactose receptor [68, 69]. PGalN and PManN, however, decreased the hepatic uptake of liposomes whereas PGal and PMan did not affect the uptake. The author considered that sugar residue of synthesized glycolipid was not exposed on the liposomal surface. The author examined the lectin-induced aggregation of liposomes. An increase of turbidity was observed on the addition of lectin to a suspension of LacCer-liposomes, while that of PGalN- and PGal-liposomes was not observed, which indicates that the galactose



residue in PGalN or PGal is not positioned above the phospholipid choline groups and lectin is inaccessible to this residue. Namely, the sugar residue in aminoglycolipids or neutral-glycolipids synthesized here is not exposed on the liposomal surface, and the liver cells cannot recognize it, thus the clearance of liposomes is not affected in rats. Disaccharides, polysaccharides or glycolipids with appropriate spacers between sugar moiety and the hydrophobic group seem to be a necessary for effective delivery of liposomes to the liver [70].

*Effect of positive surface charge density on the clearance of liposomes*

The clearance and the tissue distribution of intravenously injected cationic liposomes were affected by zeta potential of liposomes. The *in vivo* behavior of the cationic liposomes with a zeta potential below +10 mV was comparable to that of neutral Control-L, while the cationic liposomes with a zeta potential of about +15 mV remained in the blood longer and accumulated in the liver less than the Control-L. The liposomes with zeta potential above +20 mV were cleared from blood circulation similarly as the Control-L. Some investigators have shown that the *in vivo* behavior of cationic liposomes was the same as that of neutral liposomes [71, 72]. Their cationic liposomes contained only 5 mol% of charged lipid and the zeta potential of their liposomes would be little different from that of neutral liposomes. Cationic liposomes containing 10 mol% of stearylamine were reported to remain in the blood longer than neutral liposomes [15, 73]. These results agree with those obtained here. Cationic liposomes containing around 50% of basic lipid (such as lipofectin™) were reported to accumulate in the liver rapidly after intravenous injection [74]. The experiment also showed that cationic liposomes with a zeta potential of 25 mV or above accumulated in the liver.

Liposomes with an optimum positive charge showed RES-avoiding ability and further increase of the charge on the surface resulted in recovery of the hepatic uptake. The optimum value of zeta potential to prolong the circulation time of liposomes was +15 mV. In contrast, the uptake of liposomes by RES also decreased with PEG

coating and reached a plateau at a certain content of PEG-lipid[75].

*Plasma protein associated with liposomes*

Serum protein binding to liposomes is an important factor in the liposomal uptake by the liver or spleen [52, 54, 55, 76-81]. Liposomes associating with large amounts of protein were rapidly eliminated from the blood circulation. The author investigated the protein association with cationic liposomes using a spin column procedure. The amount and sort of protein associated with cationic liposomes was little different from that of Control-L. Accordingly, blood circulation time did not correlate with amount of proteins associated with cationic liposomes. Consequently, RES-uptake of cationic liposomes is controlled by a factor other than serum opsonin association. Nicholas et al. indicated that erythrocytes are involved in the suppression of hepatic uptake of dipalmitoylphosphatidylcholine-based liposomes containing stearylamine [82].

**Conclusion**

In conclusion, aminoglycolipids but not neutral-glycolipids decrease the uptake of liposomes by the liver and prolong the time spent in the blood circulation. The liposomal surface charge density is an important factor determining the fate of liposomes in blood circulation. It was found that a zeta potential of about +15 mV was optimal in prolonging the blood circulation of liposomes. Enhanced delivery of drugs to the liver is also possible with cationic liposomes of large basic lipid content. In this way, cationic liposomes can be used as RES-avoiding and RES-targeting carriers by control of the positive charge on the liposomal surface.



# Chapter 3

## The mechanism of the hepatic uptake of cationic liposomes

In the preceding chapter, the author showed that the cationic liposomes can be used as both RES-avoiding and RES-targeting drug carriers. The reticuloendothelial system (RES), especially the liver, is largely responsible for the clearance of injected liposomes from the blood circulation. Many researchers have reported that the interactions between liposomes and blood components, especially serum components, play a mediating role in liposomal uptake by RES [54-56, 77, 80, 83, 84]. Liposomes bound to large quantities of blood proteins were found to be rapidly cleared from the circulation [80]. It is important to understand the mechanism of plasma clearance of administered liposomes so that the fate of liposome-entrapped drugs can be controlled for effective therapy. Information on the uptake mechanism of cationic liposomes is rather sparse.

The author showed that serum proteins bound to liposomes did not much change with the content of basic lipid. In this chapter, rat liver perfusion experiments were carried out to elucidate the mechanisms of the uptake of cationic liposomes. This method enables one to evaluate the effects of each blood component under the same conditions of blood flow, cell polarity and spatial architecture of hepatocytes and capillary bed.

### 3-1 Materials and Methods

*Materials*

[1-$^{14}$C]-Palmitic acid were purchased from Daiichi Pure Chemical Co. Ltd. (Tokyo, Japan). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide, hydrochloride (WSC) was obtained from Dojindo Laboratories (Kumamoto, Japan). All other chemicals were obtained as described in 1-1 and 1-2. Water was glass distilled twice.

*Synthesis of radiolabeled methyl-2-amino 6-palmitoyl glucoside*

Radiolabeled PGlcN was synthesized in the manner described in 1-1 with some modification. A mixture of methyl-2-(N-benzyloxycarbonyl)amino-2-deoxyl-D-glucoside (2 mmol), [1-$^{14}$C]-palmitic acid (2 mmol) and WSC (3 mmol) in pyridine was stirred overnight. After addition of water (10 ml), the reaction mixture was filtered and purified by column chromatography over silica gel with chloroform-ethyl acetate (1:1). The purified compound was reduced as described in 1-1.

*Preparation of liposomes*

Liposomes were prepared as described in 2-1. Multilamellar vesicles (MLVs) were prepared by extrusion [40]. Liposomes were composed of egg phosphatidylcholine, cholesterol and test lipid (PGlc, PGlcN or SA) in the desired molar ratios. The lipid mixtures in chloroform were evaporated to form thin lipid films. To prepare lipid-labeled liposomes, $^{3}$H-CHE, a non-exchangeable, non-degradable marker, was added to the lipid mixture. The thin lipid film was dried overnight *in vacuo*, then hydrated with phosphate-buffered saline (PBS, pH 7.4). The suspensions were successively extruded through polycarbonate filters of various pore sizes (0.6 and 0.2 μm pore size 5 times; vesicles with a diameter of 200 nm; $VET_{200}$ were prepared by extrusion technique). The size of the liposomes was measured by dynamic light scattering on a Photal laser particle analyzer (LPA-3100; Otsuka Electronics Co. Ltd., Japan) connected to a photon correlator (LPA-3000). According to the dynamic light scattering method, the mean diameter of the liposomes was approximately 200 nm, with homogeneous distribution as described in chapter 2 (Table 2-2). The zeta potential of liposomes with a diameter of approximately 200 nm was calculated based on Smoluchowski's equation [42] from their electrophoretic mobility in PBS (pH 7.4) at 25 °C obtained by an electrophoretic light scattering spectrophotometer (Zetasizer 4; Malvern Instruments, U. K.).



*Perfusion of rat liver*

The livers of male Wistar rats, weighing from 180 to 200 g, were perfused by the method of Mortimore et al. [85] with some modification. The rats were anesthetized by an intraperitoneal injection of Nembutal. The abdomen was opened wide, the common bile duct was cannulated with a polyethylene tube (PE-10) and the hepatic portal vein was cannulated with a polyethylene tube (PE-160). The chest was opened and the inferior vena cava was cannulated through the right atrium with a polyethylene tube (Orion AWG-12). The inferior vena cava immediately above the renal vein was then ligated to prevent leakage of the perfusate. Liposome uptake was measured by recirculating (i) and continuous flow methods (ii)

(i) The liver was perfused at a rate of 25 ml/min. in the physiological direction, using a peristaltic pump (ATTO SJ-1215) with a combined bubble trap-depulser located between the pump and the portal cannula. The reservoir was 80 ml of liposomal suspension containing 5 μmol of total lipid in 10 mM HEPES/Hanks buffer (pH 7.4). This was stirred gently, maintained at 37 °C, and continuously bubbled with 95%-oxygen/5%-carbon dioxide. To prepare perfusate containing erythrocytes, the cells were separated from blood and packed erythrocytes were added to the perfusate. The liver was initially flushed with 200 ml of buffer before being perfused with vesicle suspension in a closed loop. The perfusion was continued for 1 hour, during which 200 μl aliquots of liposomal suspension were taken from the reservoir at 5 minute intervals. When the perfusate contained blood or erythrocytes, the samples were decolored by 100 μl of 30 % $H_2O_2$. The liposome concentration in the perfusate was determined by using $^{3}$H-CHE as the lipid marker. Finally, the liver was flushed with 150 ml of buffer and collected. To determine the amounts of the liposomes in liver, about 50 mg of tissue was dissolved in Soluene-350 and neutralized with HCl, then Clear-sol I was added. The tissue samples were examined in triplicate. The radioactivity levels in the samples were determined on a scintillation counter (LS5000TA; Beckman U.S.A.).

(ii) The liver was perfused at a rate of 25 ml/min. The perfusate was a vesicle suspension of 50 μM total lipid in 10 mM HEPES/Hanks buffer (pH 7.4), maintained

at 37 °C, and continuously bubbled with 95%-oxygen/5%-carbon dioxide. The liver was initially flushed with 150 ml of the buffer before being perfused. Samples of the outflow passed through the liver were taken at regular intervals. The extract ratio ($E$) was calculated from Eqn 2,

$$E(\%) = \frac{C_{in} - C_{out}}{C_{in}} \quad (2)$$

where, $C_{in}$ and $C_{out}$ are the lipid concentrations of the inflow and the outflow, respectively. The author confirmed that the liposomes did not bind to the polyethylene tube. The viability of the liver was checked by measurement of its bile flow (>4 µl/min).

*Zeta potential of rat erythrocytes, hepatocytes and Kupffer cells*

Rat erythrocytes were washed three times with 10 mM HEPES/Hanks buffer (pH 7.4) and dispersed in the same buffer. Suspensions of rat liver cells were prepared by perfusion of the liver with collagenase [86, 87]. The liver perfusion procedure was described above. The liver was perfused first with preperfusion buffer ($Ca^{2+}$ and $Mg^{2+}$-free HEPES-buffered saline containing 0.5 mM EGTA, pH 7.2) for 10 minutes and then with HEPES-buffered saline containing 5 mM $CaCl_2$ and 0.05 % (w/v) collagenase (type I) (pH 7.5) for 10-20 minutes. The perfusion rate was maintained at 20 ml/minute. After the perfusion, the liver was excised and the capsule membranes were removed. The cells were dispersed in ice-cold 10 mM HEPES/Hanks buffer (pH 7.4). The dispersed cells were filtered through cotton mesh sieves, then centrifuged at 50 g x 1 minute. The pellets containing hepatocytes were washed twice with Hanks buffer by centrifugation at 50 g x 1 minute. The supernatant containing nonparenchymal cells was similarly centrifuged two more times. The resulting supernatant was then centrifuged twice at 200 g for 2 minutes. Kupffer cells were subsequently purified from the nonparenchymal cells by counterflow centrifugal elutriation (SRR6Y rotor with the himac CR21, Hitachi Co., Tokyo, Japan) [88]. The zeta potential of cells was calculated as described above.



*Binding of liposomes to erythrocytes*

Rat erythrocytes were washed three times with 10 mM HEPES/Hanks buffer (pH 7.4) and dispersed in the same buffer. Erythrocyte suspensions were warmed at 37 °C, then mixed with the suspension of liposomes labeled with $^3$H-CHE (hematocrit, 1 %). After a 5-minute incubation at 37 °C, the suspension was centrifuged (300 g x 1 minute) and the radioactivity level in the supernatants was determined in the Beckman LS5000TA scintillation counter. Erythrocyte suspensions with a hematocrit of 1 % contained 1 x $10^8$ erythrocytes/ml.

## 3-2 Results

*Perfusion of rat liver*

The time courses of liposomes in the perfusate during recirculating liver perfusion are shown in Fig. 3-1. The radioactivities of neutral Control-L and PGlc-liposomes in the perfusate decreased negligibly. On the other hand, that of cationic PGlcN- and SA-liposomes rapidly decreased over 10-20 minutes, then gradually increased. This is discussed later. Fig. 3-2 shows the hepatic uptake of liposomes after 1 hour recirculating perfusion as a function of the zeta potential of liposomes. Neutral liposomes were slightly taken up by the liver, and higher positively charged liposomes presented a larger uptake by the liver in the buffer. Cationic PGlcN20-L were more accumulated than Control-L in the liver. These results are in conflict with those found *in vivo* as shown in chap-

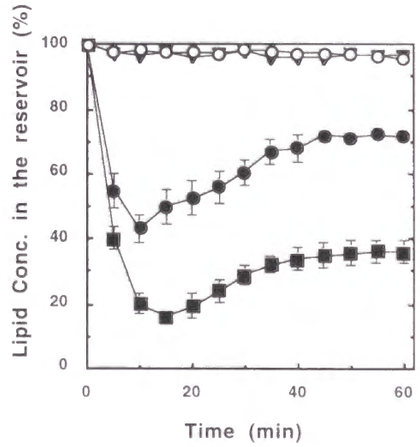


**Fig. 3-1**

Time course of the lipid concentration of the perfusate in 10 mM HEPES/Hanks buffer during the recirculating perfusion of the rat liver with liposomes. The liposomes labeled with $^3$H-CHE were prepared by extrusion ($VET_{200}$). The initial lipid concentrations were 62.5 µM and each value is expressed as a percentage ± S.D. of the initial $^3$H-CHE radioactivity. n=3.
Liposomes: (○), EPC:Chol(8:2);
(▼), EPC:Chol:PGlc(6:2:2);
(●), EPC:Chol:PGlcN(6:2:2).
(■),EPC:Chol:SA(6:2:2).

ter 2 (Figs. 2-6 ~ 2-10), suggesting that interactions between liposomes and blood components play a role in liposomal uptake. Therefore, the effects of blood on the hepatic uptake were investigated. In the presence of rat blood in the perfusate, the hepatic uptake of neutral Control-L was enhanced, while that of cationic PGlcN20-L was suppressed as shown in Fig. 3-3. To examine the effect of the blood on the hepatic uptake of PGlcN20-L in more detail, rat serum and erythrocytes were separately added to the perfusate. Rat serum of 1.25 %v/v (the same volume as that used in the above experiment) in the perfusate led to a slight decrease of hepatic uptake of PGlcN20-L. Rat erythrocytes (hematocrit, 1 %) suppressed the hepatic uptake significantly as shown in Fig. 3-3.

The results of the continuous flow method are shown in Fig. 3-4. The results of the continuous flow method and recirculating methods were similar. The uptake of Control-L and PGlcN20-L in buffer reached a steady state within about 60 and 200

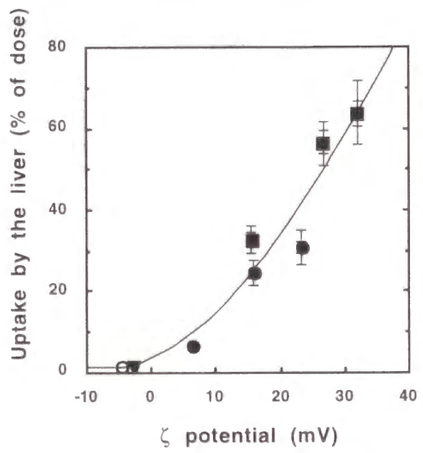


**Fig. 3-2**
The hepatic uptake of liposomes after 1 hour recirculating perfusion in rat. The liposomes labeled with $^3$H-CHE were prepared by extrusion ($VET_{200}$). The liver was perfused for 1 hour at a dose of 5 μmol of total lipid and each value is expressed as a percentage of ± S.D. of the administered $^3$H-CHE radioactivity. n=3.
(○), EPC:Chol(8:2); (▼), EPC:Chol:PGlc(6:2:2); (●), PGlcN-liposomes; (■),SA-liposomes.

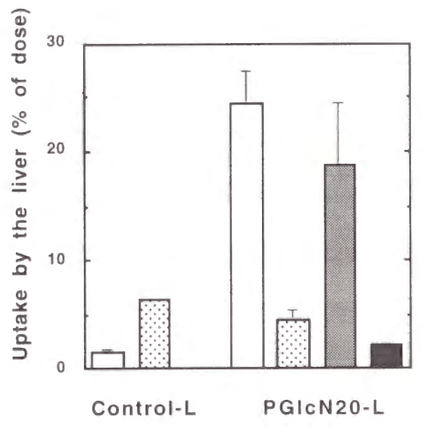


**Fig. 3-3**
Effects of rat whole blood, serum, and erythrocytes on the hepatic uptake of liposomes after 1 hour recirculating perfusion in rat. The liposomes labeled with $^3$H-CHE were prepared by extrusion ($VET_{200}$). The liver was perfused at a dose of 5 μmol of total lipid and each value is expressed as a percentage of ± S.D. of the administered $^3$H-CHE radioactivity. n=3.
(▭), 10 mM HEPES/Hanks buffer. In the same buffer containing: (▭), 2.5 v/v% rat whole blood; (▭), 1.25 v/v% rat serum; (▭), rat erythrocytes (hematocrit, 1 %)



seconds, respectively. In the steady state, extract ratios (E) of control and PGlcN-liposomes in buffer were 2.5 ± 0.4 % and 17.1 ± 0.1 %, respectively. In perfusate containing blood, the extract ratio (E) of Control-L was 5.0 ± 0.9 %. Thus, the uptake of Control-L was enhanced by addition of the rat blood. In buffer containing blood or erythrocytes, the uptake of PGlcN-liposomes reached a steady state within about 60 seconds and the extract ratios (E) were 1.6 ± 0.1 % and 2.2 ± 0.3%, respectively.

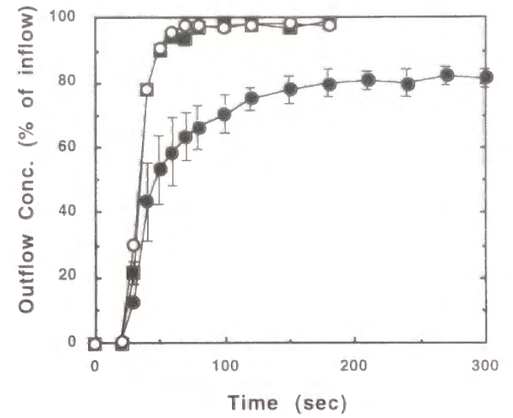


**Fig. 3-4**
Initial uptake of liposomes by constant flow perfusion of rat liver. The liposomes labeled with $^3$H-CHE were prepared by extrusion ($VET_{200}$). The liver was perfused with liposomal solution at 50 μM as a total lipid concentration and the lipid concentration in the outflow was measured. Each value is expressed as a percentage ± S.D. of the $^3$H-CHE radioactivity of the inflow. n=3.
(○), EPC:Chol(8:2)-liposomes in 10 mM HEPES/Hanks buffer; (●), EPC:Chol:PGlcN(6:2:2)-liposomes in the same buffer; (■), EPC:Chol:PGlcN(6:2:2)-liposomes in the same buffer containing rat erythrocytes (hematocrit = 1 %)

The time courses of cationic liposomal concentration in the perfusate containing rat erythrocytes (Ht = 1 %) are shown in Fig. 3-5. The radioactivity of PGlcN10-L, PGlcN20-L and SA10-L in the perfusate decreased gradually. Those of SA15-L and SA-20-L decreased immediately at first, and then gradually. Fig. 3-6 shows the hepatic uptake of cationic liposomes in the presence of rat erythrocytes after 1 hour recirculating liver perfusion. The hepatic uptakes of cationic liposomes decreased in the presence of rat erythrocytes. The suppression effect of erythrocytes on the hepatic uptake of liposomes changed with surface charge density (zeta potential). The maximum suppression was obtained at the zeta potential of about 15 mV. Thereafter, the suppression effect decreased with increasing zeta potential.

*Lipid transfer*

Rebound phenomenon for the lipid concentration of cationic liposomes in the perfusate was observed as shown in Fig. 3-1. It is possible that PGlcN and SA, which

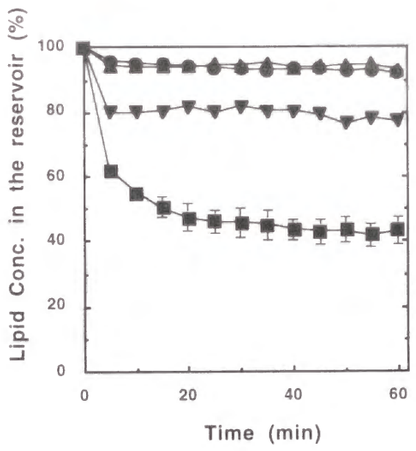


**Fig. 3-5**
Time course of the lipid concentration of the perfusate in 10 mM HEPES/Hanks buffer containing rat erythrocytes (hematocrit = 1 %) during the recirculating perfusion of the rat liver with liposomes. The liposomes labeled with $^{3}$H-CHE were prepared by extrusion ($VET_{200}$). The initial lipid concentrations were 62.5 μM and each value is expressed as a percentage ± S.D. of the initial $^{3}$H-CHE radioactivity. n=3.
Liposomes: (●), EPC:Chol:PGlcN(6:2:2); (▼), EPC:Chol:SA(7:2:1); (▲), EPC:Chol:SA(6.5:2:1.5); (■), EPC:Chol:SA(6:2:2)

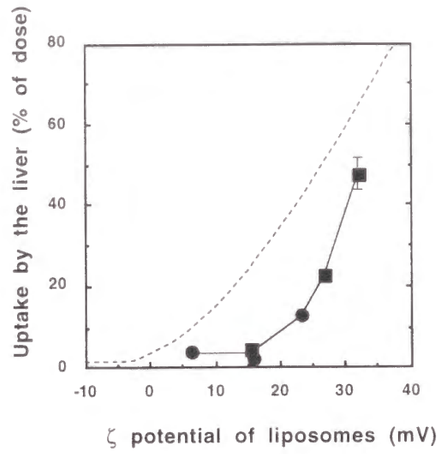


**Fig. 3-6**
The hepatic uptake of liposomes after 1 hour recirculating perfusion in rat in the presence of rat erythrocytes (hematocrit = 1 %). The liposomes labeled with $^{3}$H-CHE were prepared by extrusion ($VET_{200}$). The liver was perfused for 1 hour at a dose of 5 μmol of total lipid and each value is expressed as a percentage of ± S.D. of the administered $^{3}$H-CHE radioactivity. n=3.
(●), PGlcN-liposomes; (■),SA-liposomes; dash line denotes the hepatic uptake of liposomes in Hanks buffer.

are single-chain acyl compound like fatty acid, leaves the liposomal membrane [89-91], the electrostatic interaction between cationic liposomes and cell surface decreases, and the liposomes then dissociate from the cell surface. The dissociation of basic lipid PGlcN was examined during the rat liver perfusion using liposomes labeled with $^{3}$H-CHE and $^{14}$C-PGlcN.

The time courses of the PGlcN concentration in the liposomal membrane in the perfusate are shown in Fig. 3-7. The concentration determined by the ratio of $^{14}$C/$^{3}$H decreased to 50 % of initial liposome amount within 10 minutes, then gradually decreased in buffer, indicating that PGlcN is eliminated from the liposomal membrane and is distributed over liver cells. The ratio of $^{14}$C/$^{3}$H was also determined in the perfusate containing rat erythrocytes before and after centrifugal separation of erythrocytes. The ratio of $^{14}$C/$^{3}$H in the perfusate before centrifugation continuously de-



creased. In the erythrocyte-free supernatant of perfusate, the concentration of PGlcN in liposomal membrane was 70 % at 0 minutes as shown in Fig. 3-7, indicating that PGlcN is rapidly distributed over erythrocytes. The concentration of PGlcN in liposomal membrane decreased to about 40 % in the initial 5 minutes. The difference in the ratio of $^{14}$C/$^{3}$H between the whole perfusate and supernatant showed that PGlcN is distributed over erythrocytes and the liver cells. However, about 40 % of PGlcN remained associated with the liposomal membrane even after 1 hour of liver perfusion and the liposomes retained a positive charge.

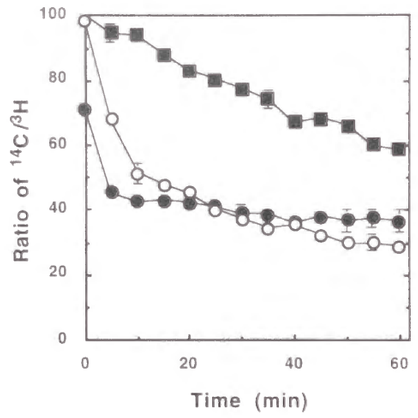


**Fig. 3-7**
Time course of the ratio of $^{14}$C/$^{3}$H during recirculating perfusion of the rat liver. The liposomes labeled with $^{3}$H-CHE and $^{14}$C-PGlcN were prepared by extrusion ($VET_{200}$). The initial lipid concentrations were 62.5 μM and the liver was perfused for 1 hour. Each value is expressed as a percentage ± S.D. of the initial PGlcN concentration in the membrane determined by the ratio of $^{14}$C/$^{3}$H. n=3.
(○), EPC:Chol:PGlcN(6:2:2)-liposomes in 10 mM HEPES/Hanks buffer; (■), EPC:Chol:PGlcN(6:2:2)-liposomes in the same buffer containing rat erythrocytes (hematocrit = 1 %), (●), EPC:Chol:PGlcN(6:2:2)-liposomes in the supernatant separated by centrifugation in the same buffer containing rat erythrocytes (hematocrit = 1 %)

*Zeta potential of cells*

The zeta potential of rat erythrocytes (-20.5 ± 2.6 mV) was lower than that of the liver cells, hepatocytes (-1.7 ± 0.6 mV) and Kupffer cells (-4.3 ± 2.2 mV).

*Binding of liposomes to erythrocytes*

The binding isotherms of the neutral and the cationic liposomes to the cells are shown in Fig 3-8. Cationic, but not neutral liposomes bound to the erythrocytes. The binding isotherms of the cationic liposomes are assumed to display a sigmoidal curve. This is because the cationic lipid PGlcN was distributed throughout the erythrocytes, and the liposomal surface charge decreased largely at low lipid concentration. When the cells and liposomes were dispersed in a medium with low ion strength using glucose, the binding increased substantially as all cationic liposomes bound to the eryth-

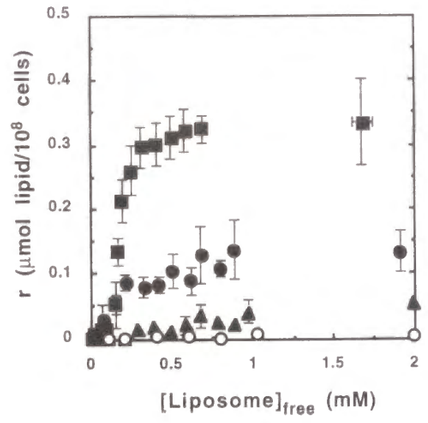


**Fig. 3-8**
Binding isotherm of liposomes to rat erythrocytes at 37 °C in 10 mM HEPES/Hanks buffer. The liposomes labeled with $^3$H-CHE were incubated with rat erythrocytes at 37 °C. After a 5-minute incubation at 37 °C, the suspension was centrifuged and the radioactivity level in the supernatants was determined.
(○), EPC:Chol(8:2); (▲), EPC:Chol:PGlcN(7:2:1); (●) EPC:Chol:PGlcN(6:2:2); (■), EPC:Chol:SA(6:2:2)

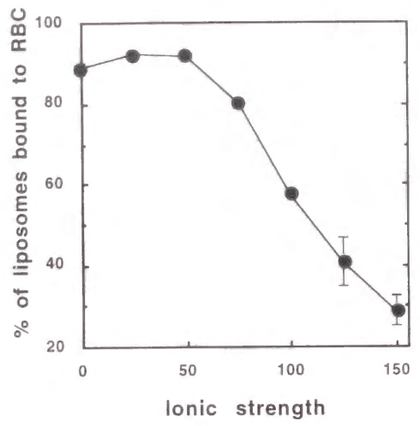


**Fig. 3-9**
Effect of ionic strength on the binding of cationic liposomes (PGlcN20-L) to the erythrocytes. The liposomes labeled with $^3$H-CHE were incubated in a buffer with rat erythrocytes at 37 °C. After a 5-minute incubation at 37 °C, the suspension was centrifuged and the radioactivity level in the supernatants was determined. Each value is expressed as a percentage ± S.D. of lipid of bound liposomes to total lipid of liposomes.

rocytes as shown in Fig. 3-9. This finding shows that the electrostatic interaction is responsible for the binding.

### 3-3 Discussion

The liver perfusion experiments demonstrated that the hepatic uptake of liposomes in buffer increased with surface positive charge of liposomes. In general, cell surfaces bear a net negative charge, and the zeta potential of Kupffer cells was -4 mV. These results suggest that the electrostatic attraction between cationic liposomes and Kupffer cells plays an important role in the hepatic uptake in the buffer [92-94]. However, this explanation contradicted the results obtained in *in vivo* experiments.

The effects of blood components on the hepatic uptake were examined. In the presence of rat whole blood or serum in the perfusate, an enhancement of hepatic uptake of neutral Control-L was observed. The author assume that this is due to the



action of opsonins [52-56]. By contrast, addition of the blood to the perfusate led to a marked decrease in hepatic uptake of the cationic PGlcN20-L. Nicholas et al. [82] also reported that hepatic uptake of dipalmitoylphosphatidylcholine-based liposomes containing stearylamine was suppressed in the presence of blood in rat liver perfusion. In the presence of rat serum, a slight decrease in the hepatic uptake of cationic PGlcN-liposomes was observed. Serum components can bind to the surface of the cationic liposomes and decrease the charge density on the liposomes, thereby causing a decrease of uptake by the liver [76]. However, these serum components are not determinant factors in the avoidance of RES by cationic liposomes, since the uptake of cationic PGlcN20-L was still higher than that of Control-L in the presence of serum. Furthermore the species and amounts of plasma protein bound to cationic liposomes were similar to Control-L as seen in the preceding chapter. Thus, the effect of serum components on the uptake was presumed to be similar between neutral and cationic liposomes.

The addition of erythrocytes influenced the uptake of cationic liposomes similarly as the addition of blood. These results show that erythrocytes play an important role in the uptake of cationic liposomes by the liver. The erythrocyte membrane has a negative charge arising mainly from sialic acid, and the zeta potential of the cells was about -20 mV in phosphate buffer [95], much lower than that of the Kupffer cells. Therefore, cationic liposomes preferentially interact with erythrocytes. Thus the author inferred a mechanism for avoidance of the uptake by the liver: The Kupffer cells could not recognize cationic liposomes bound to the erythrocytes as foreign substances, and cationic liposomes could escape from the uptake by Kupffer cells.

Why does the suppressive effect of erythrocytes on the hepatic uptake decrease with increase in the surface positive charge of liposomes? If cationic liposomes bind to the erythrocytes through electrostatic attraction, liposomes of more positive charge should bind to erythrocytes more strongly and less liposomes should be accumulated in the liver. Given that the macrophages recognize and phagocytose the erythrocytes associating with cationic liposomes as foreign substances, decrease of erythrocytes in

the perfusate should be observed. However, decrease of erythrocytes in the perfusate was not detected in the liver perfusion experiment by optical density measurement as shown in Fig. 3-10. This result showed that free liposomes are taken up by the liver but liposomes bound to erythrocytes are not.

Accordingly, the author propose the following model: The binding isotherm showed that cationic liposomes did not bind to the erythrocytes completely and free cationic liposomes existed. Free cationic liposomes interact with macrophages electrostatically and are taken up by the cells. As seen in Fig. 3-11-a, liposomes with low positive charge interact weakly with the erythrocytes and are taken

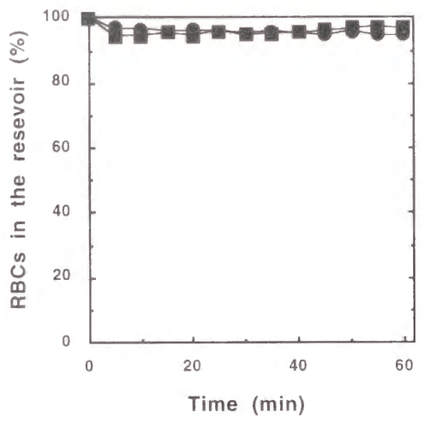


**Fig. 3-10**
Time course of the erythrocyte concentration in the perfusate during recirculating perfusion of the rat liver. The liver was perfused with liposomal suspensions in the presence of rat erythrocytes (Ht =1 %). The erythrocyte concentration in the reservoir was determined by optical density measurement at 540 nm.
(●) EPC:Chol:PGlcN(6:2:2);
(■), EPC:Chol:SA(6:2:2)

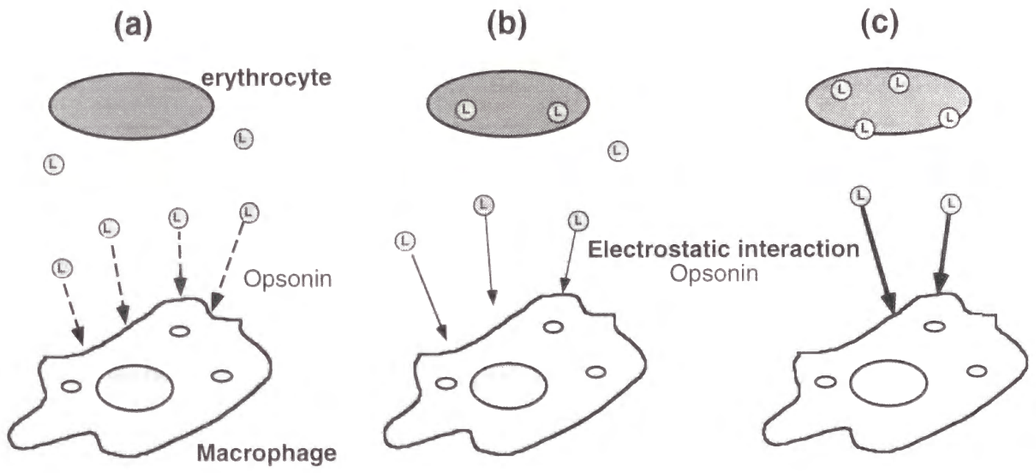


**Fig. 3-11**
Schematic diagrams for the proposed model of the interaction of cationic liposomes with erythrocytes and macrophages.
(a) cationic liposomes with zeta potentials under +10 mV,
(b) cationic liposomes with zeta potentials of about +15 mV, and
(c) cationic liposomes with zeta potentials of above +20 mV.



up by macrophages as neutral liposomes. At optimum positive charge (Fig. 3-11-b), liposomes interact with erythrocytes and the fraction of free liposomes decreases, escaping from uptake by macrophages. In the case of liposomes with high positive charge (Fig. 3-11-c), while the fraction of free liposomes decreases further, free liposomes interact with macrophages strongly, and are rapidly taken up by the cells. The rapid decrease of free cationic liposomes in medium results in the dissociation of liposomes from erythrocytes, which are again quickly taken up by macrophages. This model is thus based on the balance of electrostatic interaction of liposomes with erythrocytes and macrophages. While the number of free liposomes decreases with increase of positive charge, the interaction with macrophages increases; this leads to the optimum positive charge to escape from uptake by RES.

In the presence of erythrocytes, the fraction of cationic liposomes with high positive charge, i. e., SA20-L, disappeared immediately in the perfusate, and then decreased gradually as shown in Fig. 3-5. In the first stage, free cationic liposomes interacted with liver cells electrostatically and were cleared from the perfusate, then cationic liposomes dissociated from erythrocytes were taken up by the liver gradually.

In chapter 2, the author showed that PGlcN40-L was rapidly eliminated from the plasma within 1 hour after the intravenous injection. This is explicable based on the lipid transfer from liposomes to cells: Right after injection, PGlcN40-liposomes with a large positive charge are cleared from blood circulation, the surface positive charge and zeta potential of the liposomes decrease gradually due to PGlcN lipid transfer and approach the optimum value to escape the uptake by the liver. In a preliminary experiment, cationic liposomes containing 2-3 mol% of DPTMP (1,2-dipalmitoyl-3-trimethylammonium propane), which has two long acyl chains, remained in blood circulation a relatively long time. The zeta potential of the DPTMP-liposomes was about + 5 mV. The clearance and tissue distribution of DPTMP-liposomes with zeta potential of +15 mV were similar to those of Control-L. Therefore, lipid transfer leads to decrease in surface charge density of cationic PGlcN- or SA-liposomes avoiding

uptake by RES.

**Conclusion**

In conclusion, the liver perfusion experiments showed that cationic liposomes are taken up by the liver according to their positive charge and that this uptake is suppressed in the presence of erythrocytes. Cationic liposomes interact with erythrocytes or other blood cells electrostatically and escape from phagocytosis by macrophages, thus remaining in circulation longer with optimum zeta potential. Increase in positive charge of cationic liposomes lead to augmentation of the electrostatic interaction with macrophages.



# Conclusion

The author synthesized amino-glycolipid, PGlcN as a new basic lipid. This newly synthesized PGlcN distributed in EPC/Chol bilayers stably and gave positive charge to liposomes. Cationic liposomes containing PGlcN showed low toxicity compared with stearylamine-liposomes. Further investigations of the toxicity are needed, however, PGlcN is a good candidate to give positive charge to liposomes as drug carriers. The author successfully entrapped large amounts of negatively charged substances, SODs in liposomes by means of electrostatic interaction. This method did not result in loss of activity. The entrapment efficiency increased with increase of basic lipid concentration in liposomal membrane and cationic liposomes containing a high mol% of basic lipid achieved high entrapment efficiency. This method, based on the electrostatic interaction, will be useful for effective entrapment of the anionic proteins and polymers which cannot be entrapped by reverse phase and pH-gradient methods.

The author synthesized several aminoglycolipids. These aminoglycolipids decrease the uptake of liposomes by the liver and prolong time spent in the blood circulation. In contrast, the synthesized neutral-glycolipids had no such ability. The author demonstrated that the surface charge density of liposomes is an important factor determining the *in vivo* fate of administered liposomes. The author found that a positive charge density on the liposomal surface of around +15 mV as a zeta potential is optimal to prolong the presence of cationic liposomes in the blood circulation. Enhanced delivery of drugs to the liver using cationic liposomes containing a large amount of basic lipid is also possible. In this way, cationic liposomes can be used as RES-avoiding and RES-targeting carriers by the control of positive charge on the liposomal surface.

The liver perfusion experiments showed that cationic liposomes are taken up by the liver and that this uptake is suppressed in the presence of erythrocytes. Cationic liposomes can interact with erythrocytes electrostatically. The author proposed a model for the biphasic hepatic uptake of cationic liposomes: Cationic liposomes bind to eryth-

rocytes and escape from phagocytosis by macrophages, thus prolonging the circulation time at optimum zeta potential. Increase in positive charge of liposomes lead to augmentation of the electrostatic interaction with macrophages.

The present work provides important information for the successful application of cationic liposomes to drug delivery systems.



## Acknowledgments

The author wishes to express his sincere gratitude to Professor Dr. Tetsurou Handa for his continuous guidance in the course of this work. The author is also grateful to Professor Dr. Koichiro Miyajima, Associate Professor Dr. Katsumi Matsuzaki, and Dr. Hiroaki Komatsu for their valuable suggestions concerning this study.

The author also would like to thank Professor Dr. Kaoru Fuji, Associate Professor Dr. Koyo Nishida, and Dr. Hiroyuki Yasui for their collaboration and helpful suggestions.

The author is also indebted to Dr. Yoshio Hamashima, Mr. Changqi Sun, Ms. Megumi Fujita, Mr. Tsuneaki Tottori, Mr. Fuminori Sakurai, Ms. Erika Yoshida, and other members of Department of Physical Chemistry, Faculty of Pharmaceutical Sciences, Kyoto University, for their experimental assistance.

# References


[1] Ganapathi, R., Krishnan, A., Woodinsky, I., Zubord, C. G. and Lesko, L. J. (1980) *Cancer Res.*, **40**, 630-633.

[2] Ganapathi, R. and Krishnan, A. (1984) *Biochem. Pharmacol.*, **33**, 689-700.

[3] Rahman, A., White, G., More, N. and Schein, P. S. (1985) *Cancer Res.*, **45**, 796-803.

[4] Gregoriadis, G. and Allison, A. C. (1980) *Liposomes in Biological Systems*, **Wiley**, Chichester.

[5] Gregoriadis, G. (1988) *in Liposomes as Drug Carriers (G. Gregoriadis, ed.)*, **Ch. 1, John Wiley & Sons**, Chichester.

[6] Chance, M. L., Thomas, S. C. and Peters, W. (1978) *Nature*, **272**, 55-56.

[7] Alving, C. R., Steck, E. A., Chapman, W. L. Jr., Waits, V. B., Hendricks, L. D., Swartz, G. M. Jr. and Hanson, W. L. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 2959-2963.

[8] Senior, J., Crawley, J. C. W. and Greogoriadis, G. (1985) *Biochim. Biophys. Acta*, **839**, 1-8.

[9] Senior, J. and Gregoriadis, G. (1982) *Life Sci.*, **30**, 2123-2136.

[10] Allen, T. M. and Chonn, A. (1987) *FEBS Letters*, **223**, 42-46.

[11] Illum, L. and Davis, S. S. (1984) *FEBS Letters*, **167**, 79-82.

[12] Allen, T. M., Hansen, C., Martin, F., Redemann, C. and Yau-Young, A. (1991) *Biochim. Biophys. Acta*, **1066**, 29-36.

[13] Woodle, M. C., Matthay, K. K., Newman, M. S., Hidayat, J. E., Collins, L. R., Redemann, C., Martin, F. J. and Papahadjopoulos, D. (1992) *Biochim. Biophys. Acta*, **1105**, 193-200.

[14] Blume, G. and Cevc, G. (1993) *Biochim. Biophys. Acta*, **1146**, 157-168.

[15] Gregoriadis, G. and Neerunjun, D. E. (1974) *Eur. J. Biochem*, **47**, 179-185.

[16] Felgner, P. L., Gadek, T. R., Holm, M., Roman, R., Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M. and Danielsen, M. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 7413-7417.





[17] Felgner, P. L. and Ringold, G. M. (1989) *Nature*, **337**, 387-388.

[18] Smith, J. G., Walzem, R. L. and German, J. B. (1993) *Biochim. Biophys. Acta*, **1154**, 327-340.

[19] Adams, D. H., Joyce, G., Richardson, V. J., Ryman, B. E. and Wisniewski, H. M. (1977) *J. Neur. Sci.*, **31**, 173-179.

[20] Campbell, P. I. (1983) *Cytobios.*, **37**, 21-26.

[21] Mayhew, E., Ito, M. and Lazo, R. (1987) *Exp. Cell Res.*, **171**, 195-202.

[22] Lappalainen, K., Jaaskelainen, I., Syrjanen, K., Urtti, A. and Syrjanen, S. (1994) *Pharm. Res.*, **11**, 1127-1131.

[23] Filion, M. C. and Phillips, N. C. (1997) *Biochim. Biophys. Acta*, **1329**, 345-356.

[24] Schreier, H., Gagne, L., Bock, T., Erdos, G. W., Druzgala, P., Conary, J. T. and Muller, B. W. (1997) *Pharm. Acta Helv.*, **72**, 215-223.

[25] Szoka, F. and Papahadjopoulos, D. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 4194-4198.

[26] Mayer, L. D., Bally, M. B. and Cullis, P. R. (1986) *Biochim. Biophys. Acta*, **857**, 123-126.

[27] Mayer, L. D., Tai, L. C. L., Bally, M. B., Mitilenes, G. N., Ginsberg, R. S. and Cullis, P. R. (1990) *Biochim. Biophys. Acta*, **1025**, 143-151.

[28] Turrens, J. F., Crapo, J. D. and Freeman, B. A. (1984) *J. Clin. Invest*, **73**, 87-95.

[29] Miyata, K., Nakagawa, Y., Nakamura, M., Ito, T., Sugo, K., Fujita, T. and Tomoda, K. (1988) *Agric. Biol. Chem.*, **52**, 1575-1581.

[30] Yusa, T., Crapo, J. D. and Freeman, B. A. (1984) *J. Appl. Physiol.*, **57**, 1674-1681.

[31] Freeman, B. A., Turrens, J. F., Mirza, Z., Crapo, J. D. and Young, S. L. (1985) *Fed. Proc.*, **44**, 2591-2595.

[32] Nakagaki, M., Komatsu, H. and Handa, T. (1986) *Chem. Pharm. Bull.*, **34**, 4479-4485.

[33] Kasahara, M. and Hinkle, P. C. (1977) *J. Biol. Chem.*, **252**, 7384-7390.

[34] Dische, Z. and Borenfreund, E. (1950) *J. Biol. Chem.*, **184**, 517-522.

[35] Bartlett, G. R. (1959) *J. Biol. Chem.*, **234**, 466-468.

[36] Jonas, A. J. and Speller, R. J. (1989) *Biochim. Biophys. Acta*, **984**, 257-261.

[37] Yoshihara, E. and Nakae, T. (1986) *Biochim. Biophys. Acta*, **854**, 93-101.

[38] Oyanagui, Y. (1984) *Anal. Biochem.*, **142**, 290-296.

[39] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265-275.

[40] Olson, F., Hunt, C. A., Szoka, F. C., Vail, W. J. and Papahadjopoulos, D. (1979) *Biochim. Biophys. Acta*, **557**, 9-23.

[41] Kirby, C. and Gregoriadis, G. (1984) *Biotechnology*, **2**, 979-984.

[42] Adamson, A. W. (1967) *Physical Chemistry of Surfaces, 2nd Ed.*, **ch. 9, p. 223**, Interscience Publishers, New York.

[43] Chen, L. H., Borders, C. L. Jr., Vasquez, J. R. and Kenyon, G. L. (1996) *Biochemistry*, **35**, 7895-7902.

[44] Garza-Ramos, G., Fernandez-Velasco, D. A., Ramirez, L., Shoshani, L., Darszon, A., Gomez-Puyou, M. T. d. and Gomez-Puyou, A. (1992) *Eur. J. Biochem.*, **205**, 509-517.

[45] Ryu, K. and Dordick, J. S. (1992) *Biochemistry*, **31**, 2588-2598.

[46] Crowe, L. M., Womersley, C., Crowe, J. H., Reid, D., Appel, L. and Rudolph, A. (1986) *Biochim. Biophys. Acta*, **861**, 131-140.

[47] Tanaka, K., Takeda, T., Fujii, K. and Miyajima, K. (1992) *Chem. Pharm. Bull.*, **40**, 1-5.

[48] Westman, J., Boulanger, Y., Ehrenberg, A. and Smith, I. C. P. (1982) *Biochim. Biophys. Acta*, **685**, 315-328.

[49] Strauss, G. and Hauser, H. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 2242-2246.

[50] Harrigan, P. R., Madden, T. D. and Cullis, P. R. (1990) *Chem. Phys. Lipids*, **52**, 139-149.

[51] Hoekstra, D. and Scherphof, G. (1979) *Biochim. Biophys. Acta*, **551**, 109-121.

[52] Moghimi, S. M. and Patel, H. M. (1988) *FEBS Lett.*, **233**, 143-147.

[53] Moghimi, S. M. and Patel, H. M. (1989) *Biochim. Biophys. Acta*, **984**, 384-387.

[54] Chonn, A., Semple, S. C. and Cullis, P. R. (1992) *J. Biol. Chem.*, **267**, 18759-18765.



[55] Patel, H. M. (1992) *Crit. Rev. Ther. Drug Carrier Syst.*, **9**, 39-90.

[56] Liu, D., Liu, F. and Song, Y. K. (1995) *Biochim. Biophys. Acta*, **1235**, 140-146.

[57] Kao, K. J. and Juliano, R. L. (1981) *Biochim. Biophys. Acta*, **677**, 453-461.

[58] Dave, J. and Patel, H. M. (1986) *Biochim. Biophys. Acta*, **888**, 184-190.

[59] Derksen, J. T. P., Morselt, H. W. M. and Scherphof, G. L. (1987) *Biochim. Biophys. Acta*, **931**, 33-40.

[60] Yamaoka, K., Tanigawara, Y. and Nakagawa, T. (1981) *J. Pharm. Dyn.*, **4**, 879-890.

[61] Curatolo, W., Yau, A. O., Small, D. M. and Sears, B. (1978) *Biochemistry*, **17**, 5740-5744.

[62] Chonn, A., Semple, S. C. and Cullis, P. R. (1991) *Biochim. Biophys. Acta*, **1070**, 215-222.

[63] Wessel, D. and Flugge, U. I. (1984) *Anal. Biochem.*, **138**, 141-143.

[64] Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985) *Anal. Biochem.*, **150**, 76-85.

[65] Allen, T. M., Hansen, C. and Rutledge, J. (1989) *Biochim. Biophys. Acta*, **981**, 27-35.

[66] Poste, G., Bucana, C., Raz, A., Bugelski, P., Kirsh, R. and Fidler, I. J. (1982) *Cancer Res.*, **42**, 1412-1422.

[67] Ghosh, P. and Bachhawat, B. K. (1980) *Biochim. Biophys. Acta*, **632**, 562-572.

[68] Spanjer, H. H. and Scherphof, G. L. (1983) *Biochim. Biophys. Acta*, **734**, 40-47.

[69] Spanjer, H. H., Morselt, H. and Scherphof, G. L. (1984) *Biochim. Biophys. Acta*, **774**, 49-55.

[70] Sasaki, A., Murahashi, N., Yamada, H. and Morikawa, A. (1995) *Biol. Pharm. Bull.*, **18**, 740-746.

[71] Kirby, C., Clarke, J. and Gregoriadis, G. (1980) *Biochem. J*, **186**, 591-598.

[72] Zalipsky, S., Brandeis, E., Newman, M. S. and Woodle, M. C. (1994) *FEBS Lett.*, **353**, 71-74.

[73] Abraham, I., Goundalkar, A. and Mezei, M. (1984) *Biopharm. Drug Dispos.*, **5**, 387-398.

[74] Litzinger, D. C., Brown, J. M., Wala, I., Kaufman, S. A., Van, G. Y., Farrell, C. L. and Collins, D. (1996) *Biochim. Biophys. Acta*, **1281**, 139-149.

[75] Kilbanov, A. L., Maruyama, K., Beckerleg, A. M., Torchilin, V. P. and Huang, L. (1991) *Biochim. Biophys. Acta*, **1062**, 142-148.

[76] Tyrrell, D. A., Richardson, V. J. and Ryman, B. E. (1977) *Biochim. Biophys. Acta*, **497**, 469-480.

[77] Hoekstra, D. and Scherphof, G. (1979) *Biochim. Biophys. Acta*, **551**, 109-121.

[78] Moghimi, S. M. and Patel, H. M. (1989) *Biochim. Biophys. Acta*, **984**, 379-383.

[79] Harashima, H., Sakata, K. and Kiwada, H. (1993) *Pharm. Res.*, **10**, 606-610.

[80] Semple, S. C., Chonn, A. and Cullis, P. R. (1996) *Biochemistry*, **35**, 2521-2525.

[81] Oja, C. D., Semple, S. C., Chonn, A. and Cullis, P. R. (1996) *Biochim. Biophys. Acta*, **1281**, 31-37.

[82] Nicholas, A. R. and Jones, M. N. (1991) *Biochim. Biophys. Acta*, **1074**, 105-111.

[83] Moghimi, S. M. and Patel, H. M. (1992) *Biochim. Biophys. Acta*, **1135**, 269-274.

[84] Chonn, A., Semple, S. C. and Cullis, P. R. (1995) *J. Biol. Chem.*, **270**, 25845-25849.

[85] Mortimore, G. E., Tietze, F. and Stetten, D. Jr. (1959) *Biabetes*, **8**, 307-314.

[86] Seglen, P. O. (1972) *Exp. Cell Res.*, **74**, 450-454.

[87] Doolittle, R. L. and Richter, G. W. (1981) *Lab. Invetst.*, **45**, 558-566.

[88] Knook, D. L. and Sleyster, E. C. (1976) *Exp. Cell Res*, **99**, 444-449.

[89] Kleinfeld, A. M. and Storch, J. (1993) *Biochemistry*, **32**, 2053-2061.

[90] Margolis, L. B., Baibakov, B. A., Neyfakh, A. A. Jr., Victorov, A. V., Galkina, S. I. and Bergelson, L. D. (1984) *Biochim. Biophys. Acta*, **804**, 23-30.

[91] Horber, D. H., Ottiger, C., Schott, H. and Schwendener, R. A. (1995) *J. Pharm. Pharmacol.*, **47**, 282-288.

[92] Mutsaers, S. E. and Papadimitriou, J. M. (1988) *J. Leukoc. Biol.*, **44**, 17-26.

[93] Sanderson, N. M., Guo, B., Jacob, A. E., Handley, P. S., Cunniffe, J. G. and Jones, M. N. (1996) *Biochim. Biophys. Acta.*, **1283**, 207-214.



[94] Hui, S. W., Langner, M., Zhao, Y. L., Ross, P., Hurley, E. and Chan, K. (1996) *Biophys. J.*, **71**, 590-599.

[95] Walter, H., Tamblyn, C. H., Krob, E. J. and Seaman, G. V. F. (1983) *Biochim. Biophys. Acta*, **734**, 368-372.